Pharmaceuticals as Environmental Pollutants - Cytotoxicity and Biochemical Effects in an *in vitro* Model System for Aquatic Organisms

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von

Daniel Caminada

von

Vrin GR

Promotionskomitee

Prof. Dr. Jakob Pernthaler (Vorsitz)

Prof. Dr. Leo Eberl

PD Dr. Elena Gomez

Prof. Dr. Karl Fent (Leitung der Dissertation)

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SUMMARY

This dissertation focuses on effects of human pharmaceuticals towards fish. Residues of pharmaceuticals are regularly found in surface waters in the range of ng/L to µg/L and consequently, aquatic organisms living at polluted sites are exposed during their whole life to a variety of micro-pollutants including pharmaceuticals, but the risk of such an exposure is largely unknown. Pharmaceuticals are designed to exhibit specific modes of action on target biomolecules in humans at often low concentrations and current data indicate that most of the targets are also present in lower vertebrates. Therefore, we hypothesized that in all vertebrates the modes of action of pharmaceuticals found in the environment are mediated by the interaction with defined targets like receptors, enzymes and transporters and so are the effects. This mechanism-based approach allows the investigation of potential chronic effects in aquatic organisms.

The aim of this dissertation is to study and elucidate cellular mechanisms of pharmaceuticals in fish. First, the cytotoxicity of environmentally important pharmaceuticals in the two stable fish cell lines PLHC-1 (*Poeciliopsis lucida* hepatocellular carcinoma) and RTG-2 (rainbow trout gonadal) is assessed and correlated to their partition coefficient LogD as well as to existing data for *Daphnia magna* and fish. Further, a doxorubicin-resistant PLHC-1 subclone (PLHC-1/dox) is selected and found to be characterized by an overexpression of the transporter P-glycoprotein (ABCB1). This transporter plays a crucial role in detoxification processes and its inhibition can essentially increase the intracellular concentration of compounds. The data demonstrate for the first time the presence of a classical multidrug resistance phenotype in a non-mammalian cell system. Subsequently, several pharmaceuticals – among them the statins atorvastatin, pravastatin and simvastatin as well as gemfibrozil, sildenafil and furosemide – are found to affect the multidrug resistance mechanism in both normal PLHC-1 cells and in PLHC-1/dox cells. Finally, the presence of the peroxisome proliferator-activated receptors (PPARα, PPARβ und PPARγ) and the retinoid X receptor is detected in PLHC-1 cells. Effects mediated by fibrates (bezafibrate, clofibric acid, fenofibrate, gemfibrozil) on the expression of these receptors as well as on the activity of a peroxisomal enzyme confirm its functionality.

In conclusion, this dissertation gives new insights on the interaction of pharmaceuticals in fish cell lines with different targets like ABC-transporters and PPARs. The PLHC-1 cells were found to be a reliable *in vitro* model system for the investigation of biochemical and molecular-biological effect mechanisms in fish. Furthermore, cytotoxicity assays in fish cell lines can be a valuable tool in the risk assessment to estimate and rank the acute toxicities of compounds in order to minimize acute toxicity tests *in vivo*.

ZUSAMMENFASSUNG

In dieser Doktorarbeit werden Effekte von Arzneistoffen, welche als Verunreinigungen in Gewässern auftreten, auf Wasserorganismen untersucht. In Oberflächengewässern werden regelmässig Medikamentenrückstände im Bereich von ng/L bis hin zu µg/L gefunden. Wasserorganismen, die an belasteten Stellen leben, werden oft während ihres ganzen Lebens an eine grosse Vielfalt verschiedener, umweltrelevanter Spurenstoffe ausgesetzt. Die damit verbundenen Risiken sind jedoch wenig bekannt. Arzneistoffe haben in der Regel spezifische Wirkungen auf bestimmte definierte Biomoleküle beim Menschen - und dies oft bereits bei tiefen Konzentrationen. Aktuelle Ergebnisse deuten darauf hin, dass die meisten der Zielmoleküle auch bei einfacheren Organismen vorhanden sind. Daraus stellten wir die Hypothese auf, dass die Wirkungsmechanismen bei allen Wirbeltieren durch spezifische Interaktionen von Umweltstoffen mit Zielmolekülen wie Rezeptoren, Enzymen und Transportern erfolgen. Damit sind auch die toxischen Wirkungen analog. Dieses auf Wirkungsmechanismen basierende Konzept sollte damit erlauben, chronische Wirkungen auch bei Wasserorganismen zu ermitteln.

Diese Dissertation untersucht und beleuchtet zelluläre Mechanismen von Arzneistoffen in Fischen. Als erstes wurden zytotoxische Effekte von umweltrelevanten Arzneistoffen in der Fischzelllinie PLHC-1 (*Poeciliopsis lucida* Leberkrebszellen) und RTG-2 (Gonadenzellen von Regenbogenforellen) bestimmt und diese mit vorhandenen akuten Toxizitätsdaten für *Daphnia magna* und Fischen korreliert. Weiter gelang es uns, einen Doxorubicin-resistenten PLHC-1-Klon (PLHC-1/dox) zu selektieren. Dieser zeichnet sich durch eine erhöhte Expression von P-glycoprotein (ABCB1-Transporter) aus. Dieser Transporter spielt eine wichtige Rolle im Fremdstoffmetabolismus (Ausscheidung aus der Zelle) und seine Hemmung kann die intrazelluläre Konzentration von Fremdstoffen entscheidend erhöhen. Diese Arbeit weist zum ersten Mal nach, dass ein klassischer Multidrugresistenz-Phänotyp auch bei Fischzellen auftritt. Weiterführende Untersuchungen zeigten, dass mehrere Arzneistoffe - darunter die Statine Atorvastatin, Pravastatin und Simvastatin sowie Gemfibrozil, Sildenafil und Furosemide - Effekte auf den Multidrugresistenz-Mechanismus in normalen PLHC-1- und PLHC-1/dox-Zellen ausüben. Schliesslich wurden auch erstmals drei Peroxisomenproliferations-aktivierende Rezeptoren (PPARα, PPARβ und PPARγ) in PLHC-1-Zellen identifiziert. In der Folge untersuchten wir Effekte von Fibraten auf die

Expression der drei Rezeptoren sowie auf die Aktivität eines peroxisomalen Enzyms, welche durch Fibrate (Bezafibrat, Clofibrinsäure, Fenofibrat und Gemfibrozil) initiiert wurden. Damit konnte gezeigt werden, dass das PPAR-System in der Zelllinie funktionell ist.

Zusammengefasst gibt diese Doktorarbeit neue Erkenntnisse über Wechselwirkungen von Arzneistoffen mit verschiedenen Zielmolekülen wie ABC-Transportern und PPARs in Fischzelllinien. Die PLHC-1-Zellen erwiesen sich als zuverlässiges Modellsystem für die Untersuchung biochemischer und molekularbiologischer Mechanismen von Arzneimittelwirkungen auf Fische. Weiter könnten PLHC-1-Zellen damit als mögliche Alternativen zu *in-vivo*-Versuchen in der Risikobeurteilung akut toxischer Effekte von Substanzen Anwendung finden.

CHAPTER 1

General Introduction

General introduction

It was only in the 1970's that it became aware that pharmaceuticals occur in the environment when clofibric acid was found in the range of 0.8-2 µg/L in treated wastewater in the U.S.A. (Garrison *et al.* 1976). However, only during the last decade the improvement of analytical methods, especially the ability to determine polar compounds at trace quantities, increased the knowledge about the environmental occurrence of pharmaceuticals. Analytical studies showed that complex mixtures of environmental pollutants (xenobiotics), among them pharmaceuticals occur in surface waters like rivers, lakes and the sea and, even more surprisingly, in groundwater and drinking water (Calamari *et al.* 2003; Kolpin *et al.* 2002; Wiegel *et al.* 2004).

In contrast to analytical studies, only few studies have investigated biological effects of pharmaceuticals to aquatic organisms (Fent 2004; Lindqvist *et al.* 2005; Quintana *et al.* 2005; Roberts and Thomas 2005; Stuer-Lauridsen *et al.* 2000; Tauxe-Wuersch *et al.* 2005; Ternes 1998; Thomas and Foster 2004; Weigel *et al.* 2004). One of the reasons for the scarce knowledge is the more complex nature of the question. Ultimately, the key question is:

- Do the concentrations of pharmaceuticals found in the environment pose a risk for aquatic organisms and do they cause adverse effects?

However, this question is divided into several other questions that have to be answered first:

- What are the most relevant **pharmaceuticals** found in the environment in terms of unexpected/adverse effects on aquatic organisms?
- Which **organism(s)** and systems shall be chosen for the investigation?
- Are **acute** toxic effects occurring?
- How can **chronic** effects be measured and what are the relevant endpoints?
- Are there **differences** between mechanisms and specificities in mammals and in aquatic organisms?
- How do **compound mixtures** modulate effects?
- How do environmental concentrations **correlate** with effect concentrations?

These questions guided this doctoral thesis and therefore, they will be addressed and discussed in respect towards a better understanding of the potential risk of pharmaceuticals to the aquatic environment.

In the introduction a short overview of the current knowledge on the ecotoxicology of human pharmaceuticals is presented. Sources, pathways to the environment and the fate of pharmaceuticals are discussed followed by an overview on the concentrations in the environment. In the following the most relevant modes of action of pharmaceuticals in humans are reviewed and the occurrence of target biomolecules in aquatic organisms is described. Concluding the general introduction, known adverse effects of pharmaceuticals on non target-organisms are discussed. Our review 'Ecotoxicology of Human Pharmaceuticals' in the appendix gives more detailed information on that topic. Finally, the concept of this study is introduced and the following chapters are shortly summarized.

Sources, pathways and fate of pharmaceuticals

Pharmaceuticals are a class of chemical compounds produced to induce a beneficial effect usually on specific targets like receptors, enzymes and transporters in humans (human medicine), but also in animals (veterinary medicine). They are used to treat illnesses and diseases, but also to improve the lifestyle. About 3000 different

Table 1
Annual consumption of different classes of prescribed drugs for different countries. A top 15 sold-list is shown for every country.

	Australia, 1998 [1]		England, 2000 [2]		Germany, 2001 [3]		Switzerland, 2004 [4]	
	drug	[t/a]	drug	[t/a]	drug	[t/a]	drug	[t/a]
1	Paracetamol	295.9	Paracetamol	390.9	Acetylsalicylic acid	836.3	Paracetamol	95.2
2	Metformin	90.9	Metformin	205.8	Paracetamol	621.6	Metformin	51.4
3	Lactulose	88.1	Ibuprofen	162.2	Metformin	516.9	Acetylsalicylic acid	43.8
4	Amoxycillin	46.2	Amoxycillin	71.5	Povidine iodine	483.3	Ibuprofen	25.0
5	Ranitidine	33.7	Sodium valporate	47.5	Ibuprofen	344.9	Iopromid	6.9
6	Cephalexin	25.4	Sulphasalazine	46.4	Metamizole sodium	213.9	Salicylic acid	5.3
7	Naproxen	22.8	Mesalazine	40.4	Allopurinol	142.4	Diclofenac	4.5
8	Valproate (sodium)	20.9	Carbamazepine	40.4	Theophylline	137.4	Carbamazepine	4.4
9	Acetylsalicilic acid	20.4	Ferrous sulphate	37.5	Piracetam	121.7	Atenolol	3.2
10	Gemfibrozil	20.0	Ranitidine HCl	36.3	Amoxicillin	115.4	Metoprolol	3.2
11	Allopurinol	19.1	Cimetidine	35.7	Metoprolol	93.0	Hydrochlorothiazid	2.2
12	Sulphasalazine	18.0	Naproxen	35.1	Carbamazepine	87.6	Naproxen	1.7
13	Ibuprofen	14.2	Atenolol	29.0	Ranitidine	85.8	Ranitidine	1.6
14	Chlorothiazide	12.2	Oxytetracycline	27.2	Diclofenac	85.8	Furosemide	1.0
15	Quinine (sulphate)	11.7	Erythromycin	26.5	Penicillin V	82.5	Bezafibrate	0.8

References: 1) Khan, S. J. and Ongerth, J. E. (2004). Chemosphere 54(3): 355-67. 2) Jones, O. A., Voulvoulis, N., et al. (2002). Water Res 36(20): 5013-22. 3) Huschek, G., Hansen, P. D., et al. (2004). Environ Toxicol 19(3): 226-40. 4) ©IMS Health Incorporated or its affiliates. All rights reserved. MIDAS–02/03/05.

substances are used in the European Union (EU) such as analgesics and anti-inflammatory drugs, lipid regulators, contraceptives, antibiotics, beta-blockers, neuroactives and many others. The consumption of pharmaceuticals is substantial (Tab. 1). In countries like Australia, England and Germany, the 15 most frequently sold drugs account for hundreds of tons per year (Calamari *et al.* 2003; Huschek *et al.* 2004; Jones *et al.* 2002; Khan and Ongerth 2004). The amounts consumed in Switzerland are smaller due to its smaller population; however, the annual consumption of paracetamol reaches still 95 t per year. The consumption of pharmaceuticals (Tab. 1) may be even higher as only figures about annual sales of mainly prescribed drugs sold in pharmacies and drugstores are known. Data on sales over-the-counter are not known, nor are internet sales that may be of significant amounts for some drugs like sildenafil (the active ingredient of Viagra).

After consumption pharmaceuticals finally reach the environment (Fig. 1), either in their form by which they reach the targets within an organism to exhibit the expected effect or as metabolites. Therefore, pharmaceuticals are often excreted in their native form or as rather stable metabolites, but they may enter aquatic systems also after improper disposal (Fig. 1). Hospital wastewater, wastewater from manufacturers and landfill leachates may contain significant concentrations of pharmaceuticals (Holm *et al.* 1995; Metcalfe *et al.* 2003a; Metcalfe *et al.* 2003b). In addition, accidents in the chemicals industry may cause acute damage to ecosystems. Pharmaceuticals and their metabolites not readily degraded during the processing in sewage treatment plants (STP) are being discharged in treated effluents and finally resulting in the

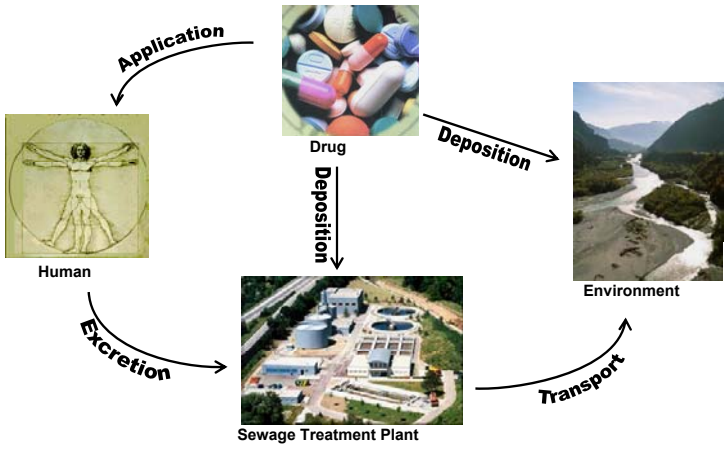

Figure 1
Pathways of human pharmaceuticals to the environment. The main route is excretion from humans and via STP to the environment. However, direct disposal to STPs or to the environment are further alternatives. At all stations, biotransformations occur (see text).

contamination of rivers, lakes, estuaries and rarely, the sea, groundwater and drinking water. Contamination of soil, runoff into surface water but also drainage may occur where sewage sludge is applied to agricultural fields. Pharmaceuticals used in veterinary medicines enter soil and aquatic systems via manure application to fields and subsequent runoff, but also directly via application in aquaculture (fish farming).

The behaviour and fate of pharmaceuticals and their metabolites in the aquatic environment is not well known (Fent 2004; Gross *et al.* 2004; Khan and Ongerth 2004; Kümmerer 2004). Their low volatility indicates that the distribution in the environment occurs primarily through aqueous transport, but also via food chain dispersal. In the wastewater treatment, two elimination processes are generally important: adsorption to suspended solids (sewage sludge) and biodegradation. The physicochemical properties, especially the pK_a, value are important factors for the fate and degradation of the compounds. In general, sorption of acidic pharmaceuticals to sludge is suggested to be not very important for their elimination from wastewater and surface water, as was demonstrated in several monitoring studies (Stackelberg *et al.* 2004; Ternes *et al.* 2004; Urase and Kikuta 2005). In contrast, basic pharmaceuticals and zwitterions can adsorb to sludge to a significant extent as shown for fluoroquinolone antibiotics (Golet *et al.* 2002).

Biodegradation is suggested to be the most important elimination process in wastewater treatment as most pharmaceuticals occur in the dissolved phase. In general, biological decomposition of micro-pollutants including pharmaceuticals increases with increased hydraulic retention time and with age of the sludge in the activated sludge treatment. However, there are considerable differences in the efficiency of the biodegradation process found for different compounds. It should be noted that a bio(re)activation of compounds may also occur. Conjugates can be cleaved during sewage treatment resulting in the release of the active parent compound as shown for estradiol (Panter *et al.* 1999; Ternes *et al.* 1999) and the steroid hormone in the contraceptive pill, 17α-ethinylestradiol (D'Ascenzo *et al.* 2003).

Elimination rates during the STP process are mainly studied by measuring the influent and effluent concentrations. As expected, they vary according to the construction and treatment technology, hydraulic retention time, season and performance of the STP. Temperature is an essential factor for good efficiencies of most STPs. Studies revealed

that elimination efficiencies of pharmaceuticals span a large range from 0-100% (Carballa *et al.* 2004; Stumpf *et al.* 1999; Ternes 1998). Very high total eliminations were found (90-100%) for propranolol, salicylic acid, ibuprofen, naproxen, ketoprofen and diclofenac (Metcalfe *et al.* 2003a; Ternes 1998; Thomas and Foster 2004). On the other hand, low removal rates (0-10%) were measured for the very hydrophilic X-ray contrast media (diatrizoate, iopamidol, iopromide, iomeprol), the anticancer drug tamoxifen (Roberts and Thomas 2005; Ternes and Hirsch 2000) and carbamazepine. The latter is hardly biodegradable independently from hydraulic retention times (Metcalfe *et al.* 2003a; Metcalfe *et al.* 2003b; Roberts and Thomas 2005; Tauxe-Wuersch *et al.* 2005).

Biodegradation still occurs in surface waters, but abiotic transformations are probably more important. Photolysis plays the most important role in the removal of diclofenac in surface water (Buser *et al.* 1998) and laboratory experiments indicate that photolysis may also be an important removal process for other pharmaceuticals like sulfamethoxazole, ofloxacin and propranolol (Andreozzi *et al.* 2003). In contrary, hydrolysis is generally negligible for environmentally relevant pharmaceuticals.

Environmental concentrations of pharmaceuticals

Despite biotic and abiotic transformations residues of pharmaceuticals and their metabolites occur in the environment (Daughton and Ternes 1999; Fent 2004; Halling-Sorensen *et al.* 1998; Heberer 2002; Kümmerer 2001, 2004). About 100 different pharmaceuticals from many classes of drugs (lipid regulators, anti-inflammatory, beta-blockers, sympathomimetics, antiepileptics, etc.) and some of their metabolites have been reported in many countries in treated sewage, rivers and creeks, seawater, groundwater and even in drinking water. An extended monitoring study in the U.S.A. detected in some sites as many as 38 out of 95 studied micro-pollutants, among them steroids, insect repellent, caffeine, antibiotics and some pharmaceuticals (Kolpin *et al.* 2002). In the river Po and river Lambro (Italy), atenolol, bezafibrate, furosemide, and antibiotics were found at all sampling sites (Calamari *et al.* 2003). Similarly, the presence of many pharmaceuticals (diclofenac, ibuprofen, carbamazepine, lipid regulators) was shown in the river Elbe (Germany) (Wiegel *et al.* 2004). A monitoring study upstream and downstream of selected towns in Iowa (U.S.A.) detected prescription drugs only frequently during low-flow conditions (Kolpin *et al.* 2004).

Environmental concentrations of pharmaceuticals occur in the low ng/L (detection limit) to µg/L range. Some data from analytical studies are compiled in figure 2 representing maximal, median and mean concentrations in treated sewage (a) and in surface waters (b). Surface water concentrations are often measured at locations near STPs. Generally, the effluents bear an about 10-fold higher concentration than surface waters. Caffeine, due to its world-wide consumption and high persistence in the environment proposed as anthropogenic marker, has been found in concentrations up to 100 µg/L in treated sewage (Weigel *et al.* 2004). Besides its use in beverages it is also part of different combinations of medicines due to its analeptic characteristics. The rather persistent clofibric acid, a metabolite of the lipid lowering agents clofibrate, etofibrate and etofyllin clofibrate, and the antiepileptic carbamazepine have been detected regularly in STP effluents, rivers and lakes and even in seawater (Buser *et al.* 1998; Weigel *et al.* 2004). In surface water, maximal concentrations of 1.2 µg/L carbamazepine and 0.55 µg/L clofibric acid were measured.

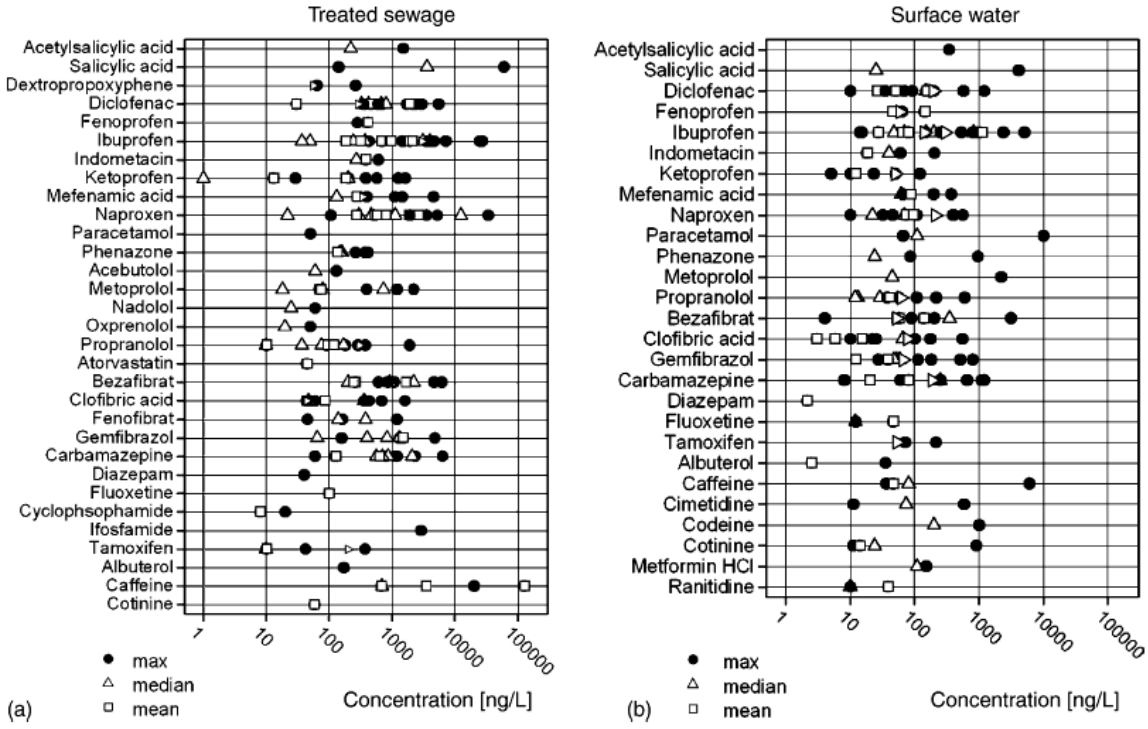

Figure 2
Concentrations of pharmaceuticals in treated wastewater (a) and surface water (b). *References*: (Andreozzi *et al.* 2003; Calamari *et al.* 2003; Gross *et al.* 2004; Halling-Sorensen *et al.* 1998; Jones *et al.* 2002; Khan and Ongerth 2004; Kolpin *et al.* 2002; Kümmerer 2004; Lindqvist *et al.* 2005; Metcalfe *et al.* 2003a; Metcalfe *et al.* 2003b; Quintana *et al.* 2005; Roberts and Thomas 2005; Stackelberg *et al.* 2004; Stuer-Lauridsen *et al.* 2000; Tauxe-Wuersch *et al.* 2005; Ternes 1998; Thomas and Foster 2004; Weigel *et al.* 2004). According to Fent et al. (2006).

Modes of action of pharmaceuticals in humans and occurrence of target biomolecules in aquatic organisms

According to their therapeutic use, pharmaceuticals are divided into different classes as non-steroidal anti-inflammatory drugs (NSAIDs), lipid lowering agents, beta-blocker, neuroactive compounds (antiepileptics, antidepressants), cytostatics and cancer therapeutics and various others. Here, only the ecotoxicologically most relevant drugs and those included in this dissertation are regarded.

Due to their broad range of applications, NSAIDs like acetyl salicylic acid, diclofenac, ibuprofen, mefenamic acid and naproxen belong to the most often used pharmaceuticals and in many countries, they are available over-the-counter. NSAIDs exhibit analgesic, antipyretic and anti inflammatory effects. Also paracetamol is often mentioned within the NSAIDs. It has only negligible anti-inflammatory activities and therefore, strictly speaking, does not belong to NSAIDs.

NSAIDs act by inhibiting either reversibly or irreversibly one or both of the two isoforms of the cyclooxygenase enzymes (COX-1 and COX-2), which catalyze the synthesis of different prostaglandins from arachidonic acid (Vane and Botting 1998). Earlier NSAIDs inhibited both COX-1 and COX-2 at different degrees, whereas new products are designed to act more selectively on COX-2, the inducible form responsible for the inflammatory reactions (Kurumbail *et al.* 1997). Recently, a third cyclooxygenase-1 variant, COX-3, has been found and characterized towards its interaction with paracetamol (Chandrasekharan *et al.* 2002). The authors argue that inhibition of COX-3 could represent a primary central mechanism by which drugs like paracetamol decrease pain and possibly fever.

In fish, several COX homologues have been found. An inducible COX-2 has been shown to be expressed in macrophages of *Onchorhynchus mykiss* and in goldfish (*Carassius auratus*) (Zou *et al.* 1999). COX-1 and COX-2 homologues were cloned from brook trout (*Salvelinus fontinalis)* ovary and a shark COX was found in *Squalus acanthias* (Roberts *et al.* 2000; Yang *et al.* 2002).

Fibrates as well as statins belong to the class of lipid lowering drugs. They are used for the treatment of hypercholesterolemia and decrease the concentration of cholesterol -

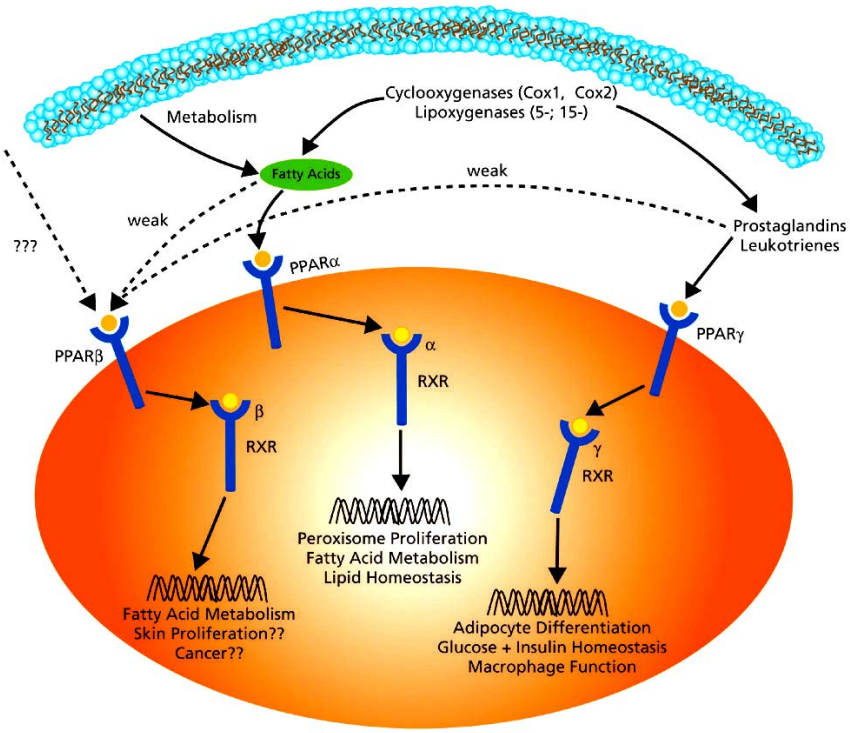

Figure 3
Simplified scheme of the PPAR-pathway. Fatty acids regulate PPARα and weakly PPARβ activity. Prostaglandins and leukotrienes regulate PPARγ and weakly PPARβ activity. Upon activation PPARs form a heterodimer with RxR and bind to PPREs of target genes.
(Taken from: ©www.sigma-aldrich.com)

and fibrates also of triglycerides - in the blood plasma. Whereas the statins inhibit the 3-hydoxymethylglutaril coenzyme A reductase (HMG-CoA) (Endo 1992), the fibrates exhibit their effects through alterations in the transcription of genes encoding for proteins controlling the lipoprotein metabolism. The target of fibrates was found to be the peroxisome proliferator-activated receptors (PPAR), mainly the PPARα isoform (Staels *et al.* 1998).

PPARs belong to the steroid/thyroid/retinoid receptor superfamily of ligand-activated nuclear transcription factors. Upon activation, they form a heterodimer with the retinoid X receptor (RxR) and bind to specific regions – the peroxisome proliferator response elements (PPRE) - of target genes. To date, three subtypes of PPAR have been described, namely PPARα, PPARβ (or PPARδ) and PPARγ. They play a key role in the lipid metabolism and the lipids homeostasis (Escher and Wahli 2000). In addition, all PPAR isoformes can participate in the regulation of inflammatory responses (Moraes *et al.* 2006). A simplified scheme of the PPAR pathway is presented in figure 3.

PPARα regulates the expression of various genes implicated in the lipid oxidation, mainly in liver and oxidative muscles, such as the heart, and is therefore involved in peroxisome proliferation (Escher and Wahli 2000; Schoonjans *et al.* 1996). Many enzymes involved in the peroxisomal β-oxidation pathway are regulated by PPARα like fatty acyl-CoA oxidase (Dreyer *et al.* 1992; Tugwood *et al.* 1992), enoyl CoA hydratase/3-OH-acyl-CoA dehydrogenase or bifunctional enzyme (Zhang *et al.* 1992) and thiolase (Lee *et al.* 1995). PPARα has also been reported to be involved in hepatocarcinogeneses in mice and rats but not in humans (Cattley *et al.* 1998).

PPARγ is a key transcription factor involved in the differentiation of adipose tissue (Rosen *et al.* 1999) and it is the molecular target for thiazolidinedione (TZD) anti-diabetic agents, which improve insulin sensitivity, glucose tolerance, and lipid homeostasis *in vivo* (Lehmann *et al.* 1995).

The least well studied isoform in terms of its biological functions and endogenous ligands is PPARβ/δ. However, it plays an important role in differentiation of epithelial tissues, fatty acid catabolism in skeletal muscle, improvement of insulin sensitivity, attenuated weight gain, and elevated HDL levels (Burdick *et al.* 2006).

Several studies have investigated PPARs in aquatic organisms. Full PPAR coding sequences have been described in fish such as plaice *Pleuronctes platessa* and gilthead sea bream *Sparus aurata* (Leaver *et al.* 1998), sea bass *Dicentrarchus labrax* (Boukouvala *et al.* 2004), zebrafish *Danio rerio* (Robinson-Rechavi *et al.* 2001), thicklipp grey mullet *Chelon labrosus* (Raingeard *et al.* 2006), and torafugu pufferfish *Takifugu rubripes* (Kondo *et al.* 2007). In addition, partial nucleotide sequences have been published for atlantic salmon *Salmo salar* (Ruyter *et al.* 1997), rainbow trout *Onchorhyncus mykiss* (Liu *et al.* 2005), brown trout *Salmo trutta* (Batista-Pinto *et al.* 2005), and goldfish *Carassius auratus* (Mimeault *et al.* 2006). Fish PPARs display an amino acid sequence identity of 46-73% to the human amphibian PPARs (Kondo *et al.* 2007).

Cytostatic compounds and cancer therapeutics are rarely found in the environment; however, they exhibit specific interactions on the cell proliferation and are already cytotoxic at low concentrations. There are different modes of actions of the different compounds. For example methotrexate acts as a potent inhibitor of the folate

dehydroreductase enzyme, which is responsible for the purine and pyrimidine synthesis (Rang *et al.* 2003). The anthracycline doxorubicin intercalates in the DNA and RNA thereby interrupts the DNA and RNA synthesis. Furthermore the inhibition of topoisomerase II and the formation of radicals leads to DNA strand breaks (Forth *et al.* 2001), and hence inhibition of cell proliferation.

Besides its cytotoxic effect, doxorubicin has been found to induce ABC-transporters especially ABCB1 (P-glycoprotein). As doxorubicin is a substrate itself of this ATP-driven transporter, cells acquire a resistance mechanisms towards this and various others structurally related substances. This phenomenon was first observed during cancer treatment and is referred to as multidrug resistance (MDR) (Juliano and Ling 1976). In aquatic toxicology, multidrug resistance came into focus when populations of aquatic organisms were observed that could survive in highly polluted environments. This phenomenon was soon termed the MultiXenobiotic Resistance mechanism (MXR) in contrast to the MDR in humans (Kurelec 1992). Presently, ABCB and ABCC transporters have been found in aquatic organisms such as clam, crab, mussel, oyster shrimp, snail, sponge, toad, worm and several fish species (Bard 2000). An analysis of the zebrafish genome revealed that over 77% of all human ABC transporters have a homologue in zebrafish (Annilo *et al.* 2006).

Ecotoxicological effects of pharmaceuticals

The best documented ecotoxicological effect represents the decline of three spieces of vultures (*Gyps bengalensis*, *Gyps indicus* and *Gyps tenuirostris*) in India and Pakistan caused by diclofenac (Oaks *et al.* 2004). High adult and subadult mortality resulting in population loss are associated with renal failure and visceral gout, the accumulation of uric acid throughout the body cavity following kidney malfunction. A direct correlation between residues of diclofenac and renal failure was reported both by experimental oral exposure and through feeding diclofenac treated livestock to vultures. Diclofenac has recently got into widespread use in these countries as a veterinary medicine to treat all kind of hoofed livestock. Furthermore, livestock that die of disease or injury are typically left for scavengers such as vultures to remove. Apart from this severe case, potential ecotoxicological effects of drug residues in the environment on wildlife are largely unknown.

Pharmaceuticals are designed to target specific metabolic and molecular pathways in humans and animals, but they often have important side effects as well. When introduced into the environment they may affect the same pathways in animals having identical or similar target organs, tissues, cells or biomolecules. For many of them homologue forms have been found in lower animals, however, there might also be differences in the specificities of targets, effects and species. For many pharmaceuticals specific modes of actions are unknown and often different modes of action may occur for a single substance. This is one reason for the difficult analysis of specific toxicities in lower animals. Despite this, toxicity experiments should be targeted and designed for specific targets of the pharmaceutical, based on the assumption of similarity of modes of actions. However, current toxicity testing is not designed in this way, as rather general and established test systems and traditional organisms according to guidelines are being used and traditional end points such as mortality are assessed.

Acute toxicity tests are regularly performed according to established guidelines (e.g. OECD, U.S. EPA, ISO) using established laboratory organisms such as algae, zooplankton and other invertebrates and fish. These data are ultimately used for ecological risk assessments. Due to animal welfare and screening purposes, *in vitro* analyses are becoming more important and they may be an alternative for the first screening and assessment of acute toxicity (see chapter 2).

For the assessment of chronic effects of pharmaceuticals to aquatic species, it is important to investigate key targets as well as different organisms. More specific investigations including analysis of possible targets of the pharmaceutical or over different life stages are lacking or have only rarely been performed. Only for the synthetic steroid ethinylestradiol (EE2) contained in the contraceptive pills life-cycle analysis have been performed. Estrogenic effects were found at concentrations as low as 1 ng/L (Brian *et al.* 2005; Länge *et al.* 2001; Parrott and Blunt 2005).

Objectives

The aim of this dissertation was the investigation and assessment of effects of human pharmaceuticals in an *in vitro* model system for fish, the stable cell line PLHC-1

(*Poeciliopsis lucida* hepatocellular carcinoma). Environmentally important pharmaceuticals as well as pharmaceuticals exhibiting specific modes of action and representing different therapeutic classes were chosen. Only little is known about effects of human pharmaceuticals to the aquatic environment. Therefore, established endpoints as well as new, mechanism-based endpoints were investigated. Further, the reliability and limitations of the used *in vitro* systems were critically evaluated and correlated to available *in vivo* data.

The areas of research included the assessment of cytotoxicity as established endpoint, investigations on the interactions of pharmaceuticals with ABC-transporters, which are involved in the efflux of xenobiotics as final step in the detoxification process, and the cloning and functional characterisation of PPARs in PLHC-1 cells.

Cytotoxicity as established endpoint

Acute toxicity tests with fish are used in the environmental risk assessment for general rankings of substances. These *in vivo* studies provide only limited information as the relevance of the findings is not related to the chronic exposure situation in the environment. The acute effect concentrations are generally a factor of $1x10^6$ higher than environmental concentrations. We hypothesize that fish cell lines can partly replace *in vivo* acute tests at least for screening purposes as most substances act by an unspecific mode of toxic action and therefore, cytotoxicity in fish cell lines should correlate with acute toxicity *in vivo*. Further, unspecific cytotoxicity has been found to correlate with the physicochemical property of the compounds such as the partition coefficient LogD; a deviation from this correlation can indicate specific cytotoxicity. We tested 34 pharmaceuticals from different classes of modes of action in two fish cell lines (PLHC-1 and RTG-2) and with two different cytotoxicity assays. We correlated the effect concentrations between the cell lines as well as between the cytotoxicity assays to first assess the reliability of fish cell lines and cytotoxicity assays. Subsequently, the partition coefficient LogD of the pharmaceuticals as well as data from *in vivo* Daphnia and fish tests was correlated to the effect concentrations of the cell lines.

Effects of pharmaceuticals on ABC-transporters

ABC-transporters are involved in the detoxification process by excreting a broad range of xenobiotics and their metabolites from the cell. The development of a resistance towards substances like cytostatics is called MultiDrug Resistance (MDR) mechanism

and is well documented in mammals; in aquatic organisms, MDR came into focus when populations of aquatic organisms were observed that were able to survive in highly polluted environments. This phenomenon was soon termed MultiXenobiotic Resistance mechanism (MXR). In order to test whether a classical MDR mechanism also exists in fish cells, we tried to select a doxorubicin-resistant PLHC-1 subclone by exposure of normal PLHC-1 cells to doxorubicin. The resulting PLHC-1 cells (PLHC-1/dox) showed the expected characteristics like overexpression of P-gp1 and resistance towards several cytostatics, and thus, represent a model system for the investigation of P-gp1 driven mechanisms.

The tested cytostatics used in the study on MDR mechanisms in the PLHC-1 cells revealed similar specificities as the one described in human cells. We concluded that other pharmaceuticals might also have similar effects in PLHC-1 cells like in human systems. We screened 33 pharmaceuticals for inhibitory effects in the wild type PLHC-1 cells as well as in the PLHC-1/dox cells. Substances that inhibit the efflux transport change also the bioavailability of substrates, thus leading to more sensitive effects e.g. modulation of cytotoxicity. We investigated such effects in co-exposure studies if inhibitors can change cytotoxicity of P-gp1 substrates in PLHC-1/dox cells.

Cloning and functional characterisation of PPARs

We proposed that pharmaceuticals act also in aquatic organisms on specific targets like enzymes, transporters and receptors. In humans, the target of fibrates like bezafibrate, clofibric acid (a metabolite of several fibrates), fenofibrate and gemfibrozil is PPARα. We proposed that all receptors belonging to the PPAR-system (PPARα, PPARβ, PPARγ and RxR) are expressed and functional in the PLHC-1 cells. Therefore, we tried to clone the receptors and investigated effects of fibrates on the expression of PPARs as well as on the activity of the peroxisomal enzyme fatty acyl CoA oxidase.

To prove the correlation of *in vitro* assays in PLHC-1 cells with findings *in vivo* we designed 14-21 days exposure experiments with fathead minnows using bezafibrate and clofibric acid as test substances. In addition to common endpoints like mortality, length, weight and numbers of eggs layed by adult fish, the expression of PPARα and the FAO-activity was measured.

The thesis is presented in 7 chapters, examining and assessing effects of pharmaceuticals in aquatic organisms:

Chapter 1: Here a general introduction to the topic and the thesis is given.

Chapter 2: Based on the fact that only little is known on effects of human pharmaceuticals in fish we assess the cytotoxicity as established endpoint in the two fish cell lines PLHC-1 and RTG-2 (rainbow trout gonadal) and correlate the data with physicochemical properties of the pharmaceuticals as well as with acute toxicity data for *Daphnia magna* and fish from the literature.

Chapter 3: This chapter describes the selection of a doxorubicin-resistant PLHC-1 subclone (PLHC-1/dox) characterized in an about 40-fold overexpression of P-glycoprotein (ABCB1). Further, the functional activity found in PLHC-1/dox is compared to wild type cells PLHC-1/wt using different cytostatics as substrates and different model inhibitors. The subclone PLHC-1/dox represents a valuable model for the investigation of transport mechanisms mediated by the P-glycoprotein.

Chapter 4: Effects of pharmaceuticals on ABC-transporters are investigated in PLHC-1/wt and PLHC-1/dox cells. In efflux assays and by fluorescent microscopy, the inhibitory potential of pharmaceuticals is assessed in both subclones using two different fluorescent model substrates. Further, the potential of pharmaceuticals to modulate cytotoxicity in PLHC-1/dox cells is assessed in co-exposure experiments.

Chapter 5: Fibrates are designed to target peroxisome proliferator-activated receptor alpha (PPARα) in humans. Here we describe the expression of all three PPAR subtypes (PPARα, PPARβ, PPARγ) and of the retinoid X receptor (RxR) that is required for a functional response by the PPAR pathway. In addition, after exposure of fibrates to PLHC-1 cells, an induction of the expression of PPARα and PPARγ is found as well as an increased activity of the peroxisomal enzyme fatty acyl oxidase that is regulated by PPARα and that is involved in the β-oxidation pathway of fatty acids.

Chapter 6: This chapter focuses on effects of the lipid lowering agents bezafibrate and clofibric acid mainly on liver enzymes in fish (fathead minnows) after 14 and 21 days of exposure. We focused particularly on peroxisome proliferator-activated receptors and

related enzymes. In addition, we monitored reproductive parameters such as egg production and vitellogenin induction to investigate the hypothesis, whether potential adverse effects of these human pharmaceuticals may be also linked to reproductive toxicity. We investigated whether both the expression of PPARα and the fatty-acyl oxidase (FAO) activity were affected in liver of fathead minnows and compared the effects to those found in PLHC-1 cells.

Chapter 7: Here, a general conclusion and outlook is given.

This thesis reveals new grounds and insights towards a mechanism-based approach in the evaluation of ecotoxicological effects of pharmaceuticals in aquatic organisms. For the first time the expression of PPARs was demonstrated in conjunction with its functionality in an *in vitro* system for fish, thus allowing further investigations of their mechanism, function and regulation. *In vivo* exposure assays in fathead minnow with two fibrates showed that these substances can have slight effects on the activity of peroxisomal enzymes after exposure for 14-21 days. Efflux transporters from the ABC-family play an important role in the elimination of compounds from cells. A doxorubicin-resistant PLHC-1 subclone characterized by an overexpression of P-gp1 (ABCB1) was selected. Effects of pharmaceuticals on efflux transporters, especially P-gp1, are described. The modulation of these transporters changes the intracellular concentration of a compound, thus leading to effects at lower concentrations. Finally, we showed that the cytotoxicity of pharmaceuticals in two *in vitro* model systems correlates well with their physicochemical properties and with *in vivo* data. Thus, *in vitro* cytotoxicity assays in fish cell lines can be a valuable tool in the risk assessment to estimate and rank the acute toxicity of compounds in order to minimize acute toxicity tests *in vivo*.

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CHAPTER 2

Cytotoxicity of pharmaceuticals found in aquatic systems: comparison of PLHC-1 and RTG-2 fish cell lines

Daniel Caminada[a,b], Claudia Escher[c], Karl Fent[b,c]

[a] University of Applied Sciences Northwestern Switzerland, School of Life Sciences, Institute of Ecopreneurship, St. Jakobs-Strasse 84, CH 4132 Muttenz, Switzerland;

[b] University of Zürich, Institute of Plant Biology, Division of Limnology, Seestrasse 187, CH-8802 Kilchberg, Switzerland;

[c] Swiss Federal Institute of Technology (ETH), Department of Environmental Sciences, CH-8092 Zürich, Switzerland

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Abstract

There is need for a better understanding of the ecotoxicity of pharmaceuticals present in aquatic systems as only little is known about their potential acute and chronic toxicity to aquatic organisms. In our work we evaluated the *in vitro* cytotoxicity of 34 common pharmaceuticals from different classes and with different modes of action using the mitochondrial thiazolyl blue tetrazolium bromide (MTT) reduction and neutral red (NR) uptake assays in the two fish cell lines, PLHC-1 and RTG-2. Cytotoxicity was found for 21 pharmaceuticals with EC50-values ranging from 2.1 µM (1.14 mg/L) (doxorubicin) to 8.66 mM (1200 mg/L) (salicylic acid). There was no significant difference between the MTT and NR assays except for an increase in absorption observed with four pharmaceuticals at low concentrations in the MTT assay with PLHC-1 cells indicating a hormesis effect. The comparison of the cell lines revealed that the PLHC-1 cell line was slightly more sensitive than the RTG-2 cell line, showing cytotoxicity at smaller concentrations. The cytotoxicity of pharmaceuticals showed a correlation with their LogD values at physiological conditions (pH 7.0). A correlation between the *in vitro* data and *in vivo* data was found for *Daphnia magna*, but not for fish due to insufficient and heterogeneous data. Our work provides an indication that *in vitro* cytotoxicity assays with fish cell lines could be suited for the first screening of the acute *in vivo* toxicity of pharmaceuticals, thereby contributing to the reduction of *in vivo* experiments. Further investigations with a larger set of pharmaceuticals are needed to strengthen the reliability of the assays and to validate the correlation with *in vivo* data.

***Key words**:* Pharmaceuticals, cytotoxicity, fish cell lines, comparison *in vitro-in vivo*, aquatic environment, REACH

Introduction

Pharmaceuticals have frequently been detected in their parent form or as metabolites in effluents of sewage treatment plants, surface waters, ground waters and even in the sea (for reviews (Daughton and Ternes 1999; Fent *et al.* 2006; Halling-Sorensen *et al.* 1998)). Several monitoring studies have shown that many pharmaceuticals from different therapeutic classes are present in surface waters with median concentrations in the range of ng/L up to µg/L (Calamari *et al.* 2003; Kolpin *et al.* 2002; Wiegel *et al.* 2004). At present, little is known about their potential adverse effects on aquatic organisms and ecosystems, despite their ubiquitous occurrence and high biological activity (Fent *et al.* 2006).

Acute toxicity tests with algae, *Daphnia* and fish are widely used for assessing the potential impact of pharmaceuticals on the environment, but there is a lack of appropriate chronic toxicity experiments that focus on potential long-term effects of pharmaceuticals. Moreover, toxicity tests are generally not designed for the specific modes of actions, and known side-effects in humans are not considered (Fent *et al.* 2006). However, chronic toxicity studies with diclofenac have shown that this compound induces effects on the kidneys of fish at environmentally relevant concentrations (Schwaiger *et al.* 2004) that resemble those in humans and in vultures on the Indian subcontinent (Oaks *et al.* 2004). Recently, the potential impact of pharmaceutical mixtures on aquatic life was shown in a study, where a complex mixture of 13 therapeutic drugs in the range of environmental concentrations caused a decrease in cell proliferation on human embryonic cells (Pomati *et al.* 2006).

For assessing the acute toxicity of pharmaceuticals to aquatic organisms, *in vitro* systems are rarely considered (Laville *et al.* 2004). This also holds true for chronic toxicity parameters that may be important for long-term effects. In the case of estrogenic pharmaceuticals, the recombinant yeast estrogen receptor assay (YES) has been demonstrated among other systems to be very important in the screening of pure compounds (Routledge and Sumpter 1996) and environmental samples (Desbrow *et al.* 1998). Using this assay, some widely used pharmaceuticals were identified as having estrogenic activity (Fent *et al.* in press). This *in vitro* assay has been demonstrated to be related to *in vivo* effects in fish (Kunz *et al.* 2006), and therefore *in vitro* systems may be indicative for effects *in vivo*. Furthermore, for animal welfare and

due to economic implications it is important to consider *in vitro* assays or other procedures to reduce the number of acute fish tests for assessing the ecotoxicity of chemicals. Recently, a strategy has been proposed to reduce fish numbers in acute toxicity tests of pharmaceuticals (Hutchinson *et al.* 2003).

The environmental safety assessment of pharmaceutical ingredients may require acute ecotoxicity data as a base set for new drugs (FDA-CDER 1998). The European Medicines Evaluation Agency (EMEA) proposed a draft guideline on the environmental risk assessment of medicinal products for human use (EMEA 2005). Depending on the predicted environmental concentration (PEC), the ecotoxicological assessment may consist of a base set of algal growth inhibition test, *Daphnia* reproduction test, and fish early life stage test, when the initial PEC is > 0.01 µg/L. In these guidelines, no *in vitro* assays are considered. However, with the White Paper on the strategy for a future chemicals policy in the European Union (EUROPEAN UNION 2001) emphasis is placed on the reduction of animal tests and on the development and application of non-animal *in vitro* testing.

A critical step towards the use of *in vitro* assays as models for *in vivo* animal experiments is the correlation between *in vitro* and *in vivo* activities. Several fish cell lines from different species and different organs have been used for cytotoxicity assays (Fent 2001; Segner 1998) and they have been shown to estimate the basal toxicity equally as well as mammalian cells when correlating the EC50 values (Castano and Gomez-Lechon 2005). In several cell lines and different fish species, good correlations between cytotoxicity *in vitro* and lethality *in vivo* have been reported (Brüschweiler *et al.* 1995; Castano *et al.* 1996; Fent 2001; Saito *et al.* 1991; Segner and Lenz 1993). This has been demonstrated for different compounds including phenols, toluenes, pesticides, metals, antimicrobials, organotins, some pharmaceuticals and others (Babich and Borenfreund 1987; Babin and Tarazona 2005; Brüschweiler *et al.* 1995; Castano and Gomez-Lechon 2005; Ni Shuilleabhain *et al.* 2004; Saito *et al.* 1991). Furthermore, the cytotoxicity has been assessed in mixtures of compounds and environmental samples (Babin *et al.* 2001; Castano *et al.* 1994; Hollert *et al.* 2000). High reproducibility, fast and economic use of fish cell cytotoxicity assays and potential correlation with *in vivo* acute toxicity are promising properties associated with these *in vitro* assays.

The aim of this work was to investigate the cytotoxicity of a series of 34 pharmaceuticals from different therapeutic classes and modes of action by applying the thiazolyl blue tetrazolium bromide ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MTT) (Mosmann 1983) and the neutral red (NR) assay (Borenfreund and Puerner 1985). Both assays are widely used for investigations of cytotoxicity in many different cell lines and proven for their accurate cytotoxicity assessment (Brüschweiler *et al.* 1995). Moreover, we compared both the sensitivity of the two assays and the cell lines PLHC-1 and RTG-2, and evaluated whether the cytotoxicity of a given compound was correlated with its LogD value at physiological conditions (pH 7.0) and with the acute toxicities *in vivo* of fish and *Daphnia*. Our study gives *in vitro* toxicity data on a series of important pharmaceuticals that may serve for toxicity identification and initial environmental assessment.

Materials and Methods

Chemicals

Calcium chloride, fetal bovine serum (FBS), glycine, HEPES, neutral red, paraformaldehyde, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Switzerland). Dulbeccos minimum essential medium with F-12 nutrient mixture (DMEM/F12) with and without phenol red, trypsin/ethylenediamine tetraacetic acid (EDTA), L-glutamine and penicillin G/streptomycin were obtained from LuBio Science GmbH (Switzerland).

Acebutolol hydrochloride (>99%), caffeine (≥ 98.5%), cimetidine (≥ 98%), clofibric acid (97%), fenofibrate (≥ 99%), furosemide (≥ 98%), gemfibrozil (≥ 99%), hydrochlorothiazide (≥ 99%), hydroxytamoxifen (≥ 98%), ibuprofen (≥ 98%), mefenamic acid (> 99%), metformin hydrochloride (≥ 98%), methotrexate hydrate (> 98%), naproxen (98%), phenazone (≥ 98%), paracetamol (≥ 99%), (±)-propranolol hydrochloride (≥ 99%), (*S*)-(−)-propranolol hydrochloride (≥ 99%), ranitidine hydrochloride (> 99%), salicylic acid (≥ 99%), and tamoxifen (≥ 99%) were purchased from Sigma-Aldrich (Switzerland), doxorubicin hydrochloride (≥ 99%), rofecoxib (98.7%), and sildenafil base (≥ 99%) from Sequoia Research Products Ltd. (United Kingdom), and pravastatin (98.7%) from ChemPacific Corporation (USA). Bezafibrate (≥ 99.3%), and diazepam (≥ 99%) were kindly supplied by F. Hoffmann-La Roche Ltd

(Switzerland), atenolol (≥ 99%), atorvastatin calcium (≥ 99%), carbamazepine (≥ 99%), diclofenac sodium salt (≥ 99%), fluoxetine (≥ 99%), (±)-metoprolol tartrate (≥ 99%), and simvastatin (98.7%) by Novartis International AG (Switzerland).

Stock solutions of all pharmaceuticals where soluble were prepared in DMSO at a concentration of 500 mM, otherwise substances were diluted corresponding to their solubility. For the cytotoxicity assays, stock solutions were diluted at least 50-times in the buffered cell culture medium (20 mM HEPES/pH 7.2) resulting in a maximal DMSO concentration of 2%, which has been shown not to be cytotoxic (data not shown). Further concentrations were prepared by serial dilution at a ratio of 1:1. All concentrations are nominal concentrations.

Cell culture

PLHC-1. The fish hepatoma cells PLHC-1 (*Poeciliopsis lucida* hepatoma cell) kindly supplied by L.E. Hightower (Ryan and Hightower 1994) were grown in DMEM/F12 supplemented with 5% FBS, 20 mM HEPES/pH 7.2 and 50 U ml^{-1} of penicillin G/50 µg ml^{-1} streptomycin in a humidified incubator with 5% CO_2 at 30°C. Cells were usually split every 4 days by dissociating with 0.05% (w/v) trypsin and 0.5 mM EDTA and subcultured at split ratios of about 1:6.

RTG-2. The rainbow trout gonadal cell line (RTG-2) was grown in DMEM/F12 supplemented with 5% FBS, 20 mM HEPES/pH 7.2 and 50 U ml^{-1} penicillin G/50 µg ml^{-1} streptomycin at room temperature (21±2°C). Cells were usually split every 7 days by dissociating with 0.05% (w/v) trypsin and 0.5 mM EDTA and subcultured at split ratios of about 1:3.

Cytotoxicity assays

For the cytotoxicity assays, cells were split and plated at densities of 6.0×10^5 cells ml^{-1} (PLHC-1) and 3.5×10^5 cells ml^{-1} (RTG-2) in 96-well tissue-culture microtiter plates (Huber & Co., Switzerland). Before exposure to pharmaceuticals, the sealed microtiter plates with cells were incubated for 24 h. Then, serial dilutions of pharmaceuticals prepared as described above were added in quadruplicates and the cells were incubated for another 24 h.

Wells without cells, cells with cell culture medium alone, cells with 2% DMSO (negative controls) and cells with a cytotoxic concentration of 5 mM naproxen or 0.16 mM fluoxetine (positive controls) were used as controls on each microtiter plate. The

cytotoxicity was determined in at least three independent experiments in quadruplicates for all pharmaceuticals.

MTT Assay. The MTT assay is based on the uptake of thiazolyl blue tetrazolium bromide (MTT) and its following reduction in the mitochondria of living cells to MTT formazan while dead cells are almost completely negative in this cleavage activity (Mosmann 1983). After 24 h of exposure to individual pharmaceuticals, 20 µl MTT solution (5 mg MTT ml^{-1} PBS) were added to each well. The microtiter plates were incubated for 4 h before cell culture medium with pharmaceuticals and MTT solution was removed and 200 µl DMSO were added to each well. After shaking for 10 min at 450 rpm, 25 µl Sorensen's Glycine buffer (50 mM glycine, 50 mM sodium chloride/NaOH pH 10.5) was added to each well. Absorption was measured spectrophotometrically at 540 nm on a microplate reader (GENios, Tecan, Switzerland). Cell viability was expressed as fraction of negative control (cells with medium only).

NR Assay. The NR assay is based on the uptake and accumulation of NR in the lysosomes of living cells. Damaged cells have altered uptake rates and dead cells are not able to retain the dye (Krone *et al.* 2005; Ryan and Hightower 1994). The NR assay was performed as described before (Brüschweiler *et al.* 1995; Ryan and Hightower 1994). After 24 h of exposure to individual pharmaceuticals, the medium was removed, the cells were washed with PBS, 200 µl NR solution (50 µg/ml medium) was added to each well and incubated for another 3 h. Afterwards, the NR solution was removed. NR taken up by cells was extracted from the cells and solubilised with isopropanol. Plates were read spectrophotometrically at 540 nm on a microplate reader (GENios, Tecan, Switzerland). Cell viability was expressed as fraction of negative control (cells with medium only).

Data analysis

Data were graphically and statistically evaluated with GraphPad Prism 4 (GraphPad Software, Inc. San Diego, CA). The raw data were logarithmically transformed. For comparison of the data evaluated in independent assays, the data were normalized to a cytotoxic concentration of naproxen or fluoxetine (positive controls) and to cells grown in medium only (negative control). Wherever applicable, the data were fitted with

Table 1
EC50-values and confidence intervals (in parenthesis) in MTT- and NR-assay for PLHC-1 and RTG-2 cells.

Pharmaceutical	CAS No.	Molecular weight	Log K_{OW} [a]	Log D [a] (pH 7)	MTT (PLHC-1) EC50 [mM]	NR (PLHC-1) EC50 [mM]	MTT (RTG-2) EC50 [mM]
Analgesics and anti-inflammatory drugs							
Diclofenac	15307-86-5	318.13	4.06	1.28	0.243 (0.213–0.278)	0.269 (0.243–0.299)	1.78 (1.53–2.06)
Ibuprofen	15687-27-1	206.28	3.72	1.16	1.20 (1.12–1.28)	0.834 (0.721–0.965)	5.79[b]
Mefenamic acid	61-68-7	241.29	5.33	2.42	0.393 (0.354–0.435)	0.218 (0.190–0.251)	1.60 (0.851–3.00)
Naproxen	22204-53-1	230.26	3.00	0.85	2.54 (2.31–2.80)	1.49 (1.17–1.89)	4.48 (3.53–5.70)
Paracetamol	103-90-2	151.16	0.34	0.34	> 10[c]	> 10[c]	> 10[c]
Phenazone	60-80-0	188.23	0.27	0.27	> 10[c]	> 10[c]	> 10[c]
Rofecoxib	162011-90-7	314.36	1.34	1.34	0.531 (0.451–0.625)	> 5[c]	1.27 (1.18–1.37)
Salicylic acid	69-72-7	138.12	2.06	− 1.03	7.24[b]	8.66[b]	> 10[c]
Blood lipid lowering agents (fibrates, statins)							
Atorvastatin	134523-00-5	1155.36	4.13	1.45	0.0818 (0.075–0.090)	0.0781 (0.063–0.097)	0.302 (0.27–0.34)
Bezafibrate	41859-67-0	361.82	3.46	0.03	2.60 (1.82–3.70)	1.74 (1.34–2.25)	1.23 [2]
Clofibric acid	882-09-7	214.65	2.72	-0.76	3.77 (3.13–4.53)	3.15[b]	5.00 (4.51–5.54)
Fenofibrate	49562-28-9	360.83	4.80	4.80	> 5[c]	> 5[c]	> 5[c]
Gemfibrozil	25812-30-0	250.33	4.39	2.15	0.873 (0.737–1.03)	0.843 (0.768–0.926)	3.94 (3.34–4.65)
Pravastatin	81093-37-0	424.54	1.44	− 1.22	5.50[b]	6.03[b]	> 10[c]
Simvastatin	79902-63-9	418.56	4.42	4.41	0.0645 (0.0564–0.0801)	0.173 (0.076–0.394)	0.144 (0.133–0.154)
β-Blocker							
Acebutolol	37517-30-9	372.89	1.95	− 0.11	> 10[c]	> 10[c]	> 10[c]
Atenolol	29122-68-7	266.34	0.097	− 2.02	> 5[c]	> 5[c]	> 5[c]
(±)-Metoprolol	51384-51-1	684.81	1.79	− 0.33	> 2[c]	> 2[c]	> 2[c]
(±)-Propranolol	525-66-6	295.80	3.10	1.00	0.158 (0.147–0.170)	0.142 (0.127–0.159)	0.0706 (0.064–0.078)
(S)-Propranolol	4199-09-1	295.80	3.10	1.00	0.152 (0.140–0.166)	0.182 (0.165–0.201)	0.102 (0.0966–0.107)
Neuroactive compounds							
Carbamazepine	298-46-4	236.27	2.67	2.67	> 2.5[c]	> 2.5[c]	> 2.5[c]
Diazepam	439-14-5	284.74	2.96	2.96	0.363 (0.326–0.403)	0.440 (0.375–0.515)	0.604 (0.440–0.830)
Fluoxetine	54910-89-3	309.33	4.09	1.31	0.0205 (0.0192–0.0219)	0.0242 (0.0211–0.0277)	0.0107 (0.0097–0.0118)
Various							
Caffeine	58-08-2	194.19	− 0.13	− 0.13	> 2.5[c]	> 2.5[c]	> 2.5[c]
Cimetidine	51481-61-9	252.34	0.072	− 0.45	> 10[c]	> 10[c]	> 10[c]
Doxorubicin	23214-92-8	543.52	3.07	1.18	0.00260 (0.00222–0.00305)	0.00217 (0.00180–0.00261)	0.00470 (0.00331–0.00667)
Furosemide	54-31-9	330.74	3.00	− 0.09	3.42 (2.83–4.13)	4.05 (3.40–4.83)	7.79 (4.80–12.6)
Hydrochlorothiazide	58-93-5	297.74	− 0.071	− 0.08	1.98 (1.79–2.18)	2.41 (2.27–2.55)	1.54 (1.17–2.03)
Metformin	657-24-9	165.62	− 2.31	− 4.31	> 2.5[c]	> 2.5[c]	> 2.5[c]
Methotrexate	59-05-2	454.44	− 0.24	− 4.90	> 0.4[c]	> 0.4[c]	> 0.4[c]
Ranitidine	66357-35-5	350.86	1.23	− 0.18	> 10[c]	> 10[c]	> 10[c]
Sildenafil	139755-83-2	474.58	2.28	2.24	> 0.25[c]	> 0.25[c]	> 0.25[c]
Tamoxifen	10540-29-1	371.51	7.88	6.20	0.0200 (0.0176–0.0228)	0.0194 (0.0167–0.0225)	0.0191 (0.0174–0.0210)
OH-Tamoxifen	68047-06-3	387.51	7.34	5.65	0.0138 (0.00114–0.167)	0.00464 (0.00407–0.00529)	0.0145 (0.0132–0.0159)

[a] Log K_{OW} and Log D-values were obtained from SciFinder. All values calculated.
[b] EC50-value deduced from normalized data.
[c] Highest tested concentrations.

the four-parameter logistic equation (Hill equation), a nonlinear regression model. In those cases where the data could not be fitted to assign an EC50-value but where cytotoxicity was evident, the EC50-value was deduced from the normalized data.

Results

The pharmaceuticals included in this study were chosen on the basis of their occurrence in the environment and their ecotoxicological potential. Thus, compounds most commonly found in the aquatic environment (Fent *et al.* 2006) and compounds exhibiting specific modes of action were selected. A series of 34 pharmaceuticals (Tab. 1) were chosen from different therapeutic classes including analgesics and anti-inflammatory drugs, blood lipid lowering drugs (fibrates, statins), β-blockers, neuroactive compounds (antiepileptic, selective serotonin reuptake inhibitor) and various others (cytostatic drugs, anti-acidic, diuretics). This broad spectrum of drugs with different characteristics allowed a comprehensive cytotoxicity assessment, and both a comparison between different cytotoxicity assays and the different fish cell lines PLHC-1 and RTG-2.

Cytotoxicity was determined for 21 pharmaceuticals using the MTT assay in PLHC-1

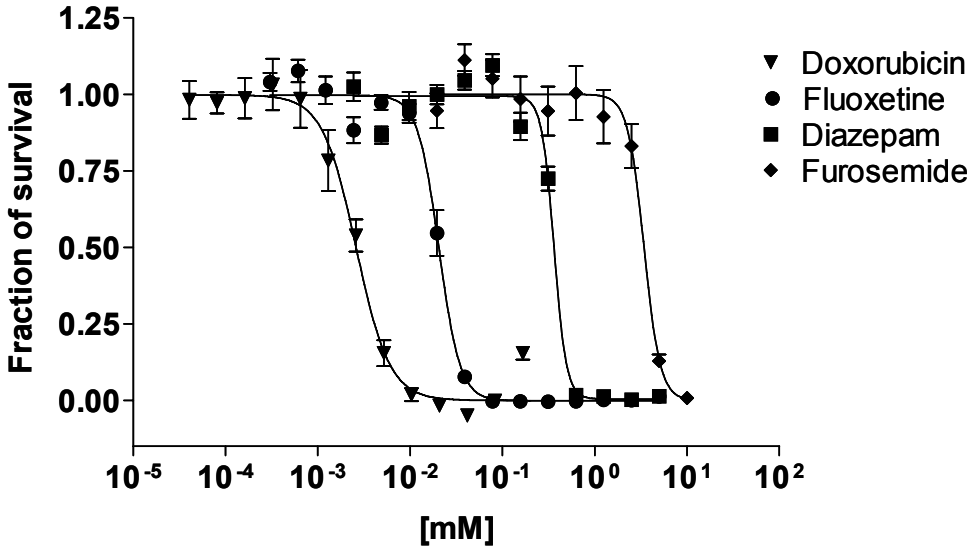

Figure 1. Ranges of cytotoxicity of doxorubicin (EC50 = 2.60 µM; 1.41 mg/L), fluoxetine (EC50 = 20.5 µM; 6.34 mg/L), diazepam (EC50 = 0.36 mM; 103.4 mg/L), and furosemide (EC50 = 3.4 mM; 1130 mg/L) observed in the MTT assay with PLHC-1 cells. Standard error of means are indicated.

cells. Acebutolol, carbamazepine, fenofibrate, and ranitidine exhibited only low cytotoxicity, i.e. the percentage of dead cells was > 50% at the highest concentration measured, and nine compounds (atenolol, caffeine, cimetidine, metformin, metoprolol, methotrexate, paracetamol, phenazone, sildenafil) showed no measurable cytotoxicity (Table 1). Figure 1 illustrates the whole range of concentrations, where complete cytotoxicity was found, as exemplified by the four pharmaceuticals doxorubicin, fluoxetine, diazepam and furosemide. Doxorubicin was the most toxic compound (EC50-value 2.1 µM ≈ 1.14 mg/L), and the diuretic furosemide with a 1,000-fold higher EC50-value (3.4 mM ≈ 1130 mg/L) was one of the least toxic compounds exhibiting a full dose response curve. The EC50-values of all compounds are given in Table 1.

Figure 2 shows the concentration-dependent cytotoxicity of pharmaceuticals of different therapeutic classes. Within each class, cytotoxicity of the various compounds varied over a considerable range. Within the class of analgesics and anti-inflammatory drugs (Fig. 2A) the EC50-values ranged from 0.24 mM (≈ 77.3 mg/L) for diclofenac to 7.24 mM (≈ 1.0 mg/L) for salicylic acid in the MTT assay. Paracetamol and phenazone did not induce cytotoxicity. The highest tested concentration for most pharmaceuticals was 10 mM (≥ 1500 mg/L), at which the DMSO concentration did not exceed 2%.

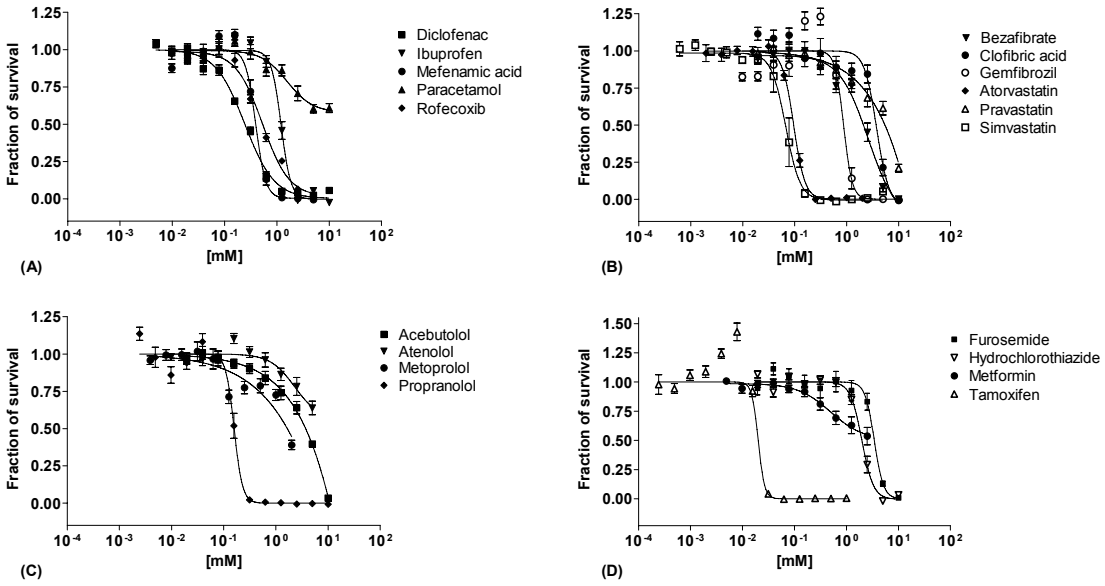

Figure 2. Cytotoxicity of different pharmaceutical classes in the MTT assay with PLHC-1 cells. A, nonsteroidal-antiinflammatory drugs: diclofenac, ibuprofen, mefenamic acid, paracetamol, rofecoxib. B, fibrates and statins: bezafibrate, clofibric acid, gemfibrozil, atorvastatin, pravastatin, simvastatin. C, β-blockers: acebutolol atenolol, metoprolol, propranolol. D, compounds with different therapeutical use: furosemide, hydrochlorothiazide, metformin, tamoxifen. Standard error of means are indicated.

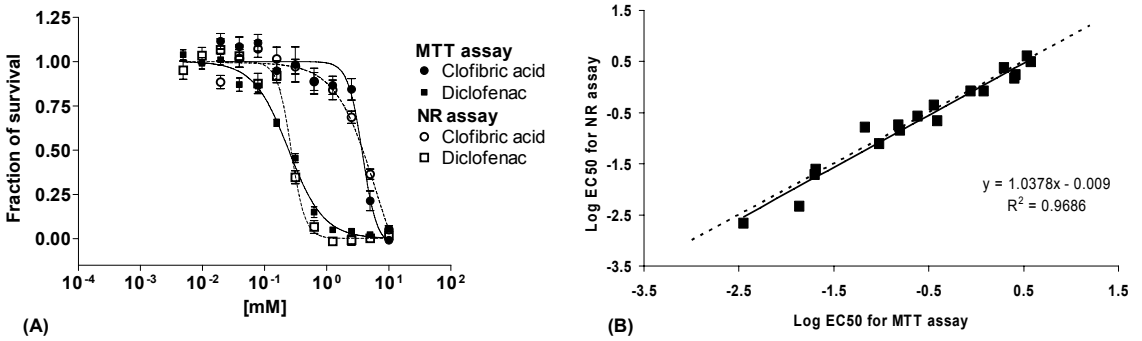

Figure 3. Comparison of MTT and NR assay. A, Cytotoxicity of diclofenac and clofibric acid. B, Linear regression of the Log EC50-values in the MTT and the NR assay. Dotted line shows a 1:1 correlation where Log EC50 (MTT) = Log EC50 (NR). Standard error of means are indicated.

Among the class of lipid lowering drugs, statins showed higher cytotoxicity than fibrates (Fig. 2B). However, pravastatin exhibited only slight cytotoxicity in the highest concentrations (estimated EC50-value of 5.5 mM ≈ 2330 mg/L). In contrast, only low cytotoxicity was observed with bezafibrate, fenofibrate, gemfibrozil and the metabolite clofibric acid (Table 1). The cytotoxicity of β-blockers (Fig. 2C) was not pronounced with the exception of propranolol (EC50-value 0.16 mM ≈ 46.7 mg/L). Acebutolol, atenolol and metoprolol showed slight cytotoxicity in the highest concentrations, but an EC50-value could not be assessed. The cytotoxicity of neuroactive compounds and various other pharmaceuticals is depicted in Figures 1 and 2D. Tamoxifen and its metabolite hydroxy-tamoxifen exhibited high cytotoxicity with an EC50-value of 20 µM (≈ 7.4 mg/L) and 14 µM (≈ 5.4 mg/L), respectively. For carbamazepine, caffeine, cimetidine, metformin, methotrexate, ranitidine, and sildenafil, no EC50-value could be established.

In order to compare the applicability and accuracy of different cytotoxicity assays, the 34 pharmaceuticals were evaluated in the NR assay. The cytotoxicities in both assays were very similar. The EC50-values were within a factor less than two (Tab. 1). However, the COX II-inhibitor rofecoxib showed cytotoxicity in the MTT, but not in the NR assay. The largest difference between the assays was found with hydroxy-tamoxifen (EC50-value of 13.8 µM and 46.4 µM in the MTT and NR assay, respectively). Figure 3A illustrates typical concentration dependent cytotoxicity curves for diclofenac and clofibric acid from both assays and Table 1 demonstrates that the EC50-values for these and other compounds are very similar. Figure 3B depicts the logarithmic EC50-values of both assays that were plotted and fitted with linear

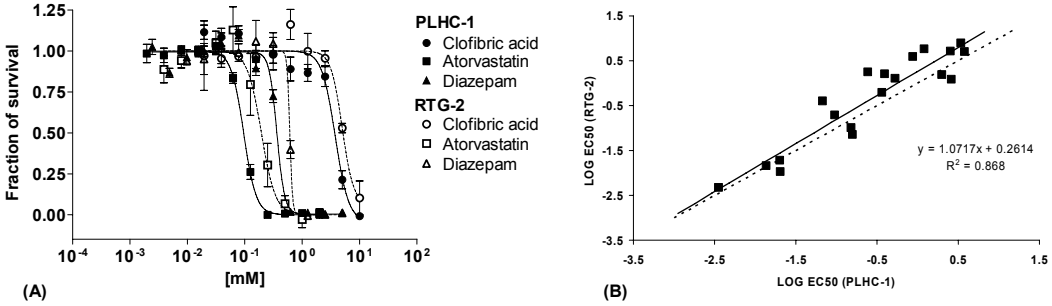

Figure 4. Comparison of cytotoxicity between PLHC-1 and RTG-2 cells. A, Cytotoxicity of clofibric acid, atorvastatin and diazepam. B, Linear regression of the Log EC50-values from PLHC-1 and RTG-2 cells in the MTT assay. Dotted line shows a 1:1 correlation where Log EC50 (PLHC-1) = Log EC50 (RTG-2). Standard error of means are indicated.

regression (Fig. 6A). The MTT and NR assays yielded a significant correlation ($R = 0.9809$; $P < 0.0001$) and a regression equation ($y = 1.0378x – 0.009$; $R^2 = 0.9686$) that was close to the ideal case of $y = x$ (y corresponds to log EC50 (MTT assay), x corresponds to log EC50 (NR assay)).

In a second series of experiments, pharmaceuticals were evaluated in rainbow trout RTG-2 cells using the MTT assay. This allowed a comparison of cell lines of different origin and cultivation conditions. Figure 4A illustrates representative cytotoxicity curves of a few compounds. There was a significant difference in the cytotoxicities of pharmaceuticals between the cell lines. Most of the EC50-values in the RTG-2 cells were slightly higher than in PLHC-1 cells which indicates a higher sensitivity of PLHC-1 cells. Exceptions were bezafibrate, propranolol, hydrochlorothiazide, and tamoxifen showing smaller values with RTG-2 cells up to a factor of two (Tab. 1). Salicylic acid was the only pharmaceutical where an EC50-value (7.24 mM) could be determined in the PLHC-1, but not the RTG-2 cell line. However, non-cytotoxic pharmaceuticals in PLHC-1 cells were also not cytotoxic in RTG-2 cells so a significant correlation was

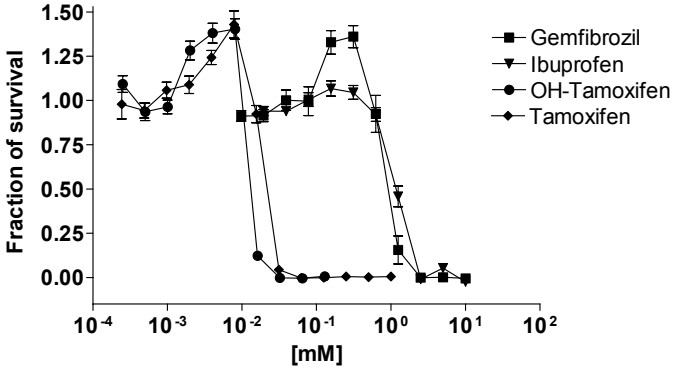

Figure 5. Hormesis effect in the MTT assay in PLHC-1 cells with gemfibrozil, ibuprofen, tamoxifen and OH-tamoxifen. Standard error of means are indicated.

found (R = 0.9317; P < 0.0001). The higher sensitivity of PLHC-1 compared to RTG-2 cells is shown in Figure 4B. The intercepts of the linear regression and the model case (x = y; x corresponds to log EC50 (PLHC-1), y corresponds to log EC50 (RTG-2)) showed a statistically significant difference (P < 0.02). The equation of the linear regression was y = 1.0717x + 0.2614 (R^2 = 0.868).

Gemfibrozil, hydroxy-tamoxifen, tamoxifen, ibuprofen, and mefenamic acid exhibited a higher absorption (increased formation of formazan in mitochondria) at non-cytotoxic concentrations (Fig. 5). Whereas the increase was only slight for ibuprofen and mefenamic acid gemfibrozil, tamoxifen and hydroxy-tamoxifen exhibited a more pronounced effect with an increase of almost 150% compared to controls. This effect was only observed in the MTT assay in the PLHC-1 cells and is considered an indication of a hormesis effect (i.e. enhanced metabolic activity like uptake of compounds at sub-toxic levels).

Figure 6 shows the correlation of the cytotoxicities of pharmaceuticals with their octanol-water partition coefficient LogD at physiological pH 7.0. The LogD coefficients were obtained from the SciFinder® Scholar (American Chemical Society) database. In the correlation analysis, the cytostatic drug doxorubicin and fluoxetine diverged apparently but also atorvastatin, propranolol and diclofenac deviated from the other compounds. The data without doxorubicin and fluoxetine showed a statistically

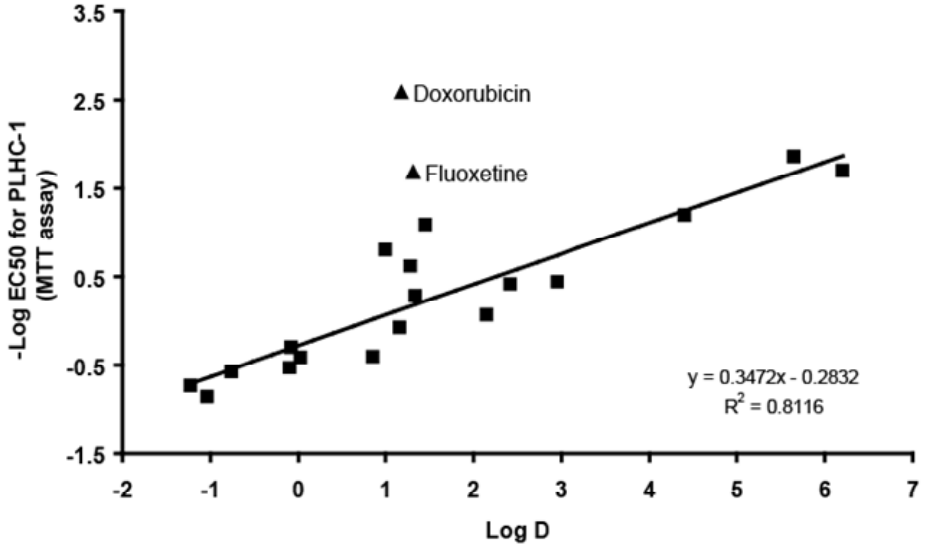

Figure 6. Correlation between Log D and –Log EC50 in PLHC-1 cells (MTT assay). Doxorubicin and fluoxetine were not included in the analysis. Log D values were obtained from the databank SciFinder.

significant correlation (R = 0.9050; P < 0.0001) between the LogD and cytotoxicity. The linear regression (y = 0.3472x - 0.2832; R^2 = 0.8116) of the data is shown in Figure 6.

In order to evaluate whether *in vitro* cytotoxicity may be indicative for acute toxicity *in vivo*, the *in vitro* cytotoxicity in fish cell lines was compared to *in vivo* data derived from reported acute toxicity data in *Daphnia* and fish. For some pharmaceuticals, several 48 h *Daphnia* data were found in the literature (clofibric acid, diazepam, diclofenac, ibuprofen, naproxen, and propranolol) so the geometric mean was taken for the correlation analysis. For caffeine, carbamazepine, cimetidine, metoprolol, paracetamol, and ranitidine the highest concentration showing cytotoxicity *in vitro* above the EC50 was taken. The *Daphnia* EC50-values were about a factor of five lower than the *in vitro* data suggesting that the fish cell lines are more sensitive. The *in vitro* cytotoxicity (negative logarithmic EC50-values) and the acute *Daphnia* toxicity (negative logarithmic EC50-values) show a significant correlation (R = 0.9530; P < 0.0001). The linear regression equation was y = 0.8159x - 0.5268 (R^2 = 0.9077) (Fig. 7A), but this is not the case with the acute toxicity data from fish where no significant correlation is found (R = 0.4852; P = 0.1552) (Fig. 7B).

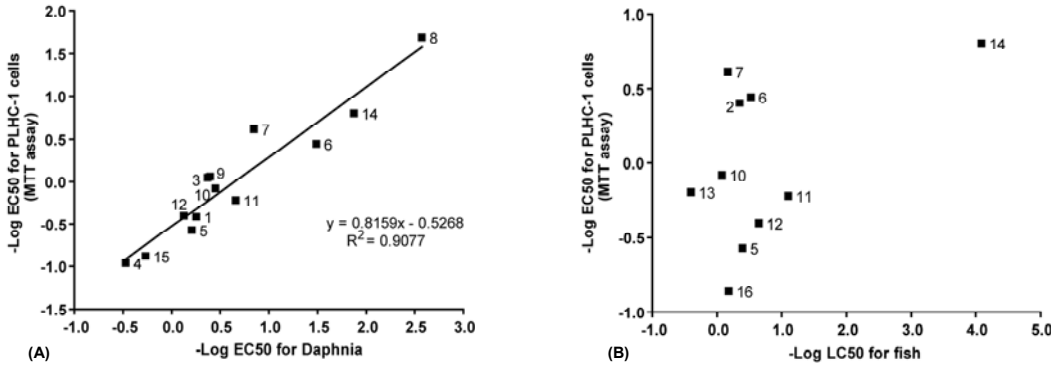

Figure 7. Comparison of *in vitro* fish cell cytotoxicity with *in vivo* data. A, correlation of cytotoxicity (-Log EC50) in PLHC-1 (MTT assay) and acute toxicity in *Daphnia* (-Log EC50) (data from: (Calleja et al., 1994; Lilius et al., 1994; Halling-Sorensen et al., 1998; Brooks et al., 2003; Cleuvers, 2003; Cleuvers, 2004; Ferrari et al., 2004; Hernando et al., 2004; Kümmerer, 2004)) B, correlation of cytotoxicity (-Log EC50) in PLHC-1 cells (MTT assay) and acute toxicity in fish (-Log LC50). *In vivo* fish data refer to different exposure times (24 h, 48 h, 96 h) and different fish species (data from (Knie et al., 1983; Halling-Sorensen et al., 1998; Ferrari et al., 2004; Kümmerer, 2004; Straub, personal communication), Novartis MSDS for diclofenac (2005) and metoprolol (2004), and Roche (2002): MSDS for Valium (Diazepam), F. Hoffmann La Roche AG, Basel). Numbers refer to the following pharmaceuticals; 1, bezafibrate; 2, caffeine; 3, carbamazepine; 4, cimetidine; 5, clofibric acid; 6, diazepam; 7, diclofenac; 8, fluoxetine; 9, gemfibrozil; 10, ibuprofen; 11, metoprolol; 12, naproxen; 13, paracetamol; 14, propranolol; 15, ranitidine; 16, salicylic acid.

Discussion

In this study, the *in vitro* cytotoxicity of 34 pharmaceuticals and their metabolites have been assessed in the two fish cell lines, PLHC-1 and RTG-2, applying the MTT and NR assay. Half-maximal cytotoxicity (EC50-value) could be determined for 21 compounds, the remaining compounds showed no or only slight cytotoxicity even at the highest concentrations. The most toxic compound found in this study was the cytostatic drug doxorubicin exhibiting an EC50-value of 21 µM (≈ 1.14 mg/L) determined with the MTT assay in PLHC-1 cells. Compared to environmental concentrations, which are in the range of ng/L to µg/L, cytotoxicity occured at concentrations of about a factor 1'000'000 higher.

Despite the slightly different cytotoxic endpoints of the two cytotoxicity assays, the effects measured in the MTT as well as in the NR assay indicate a damage of the cell membranes. The uptake and retention of MTT and NR to the mitochondria and lysosomes, respectively, are prevented. So the result was expected that both cytotoxicity assays yielded nearly the same results with a high correlation (R = 0.98). Moreover, the cytotoxicity in both cell lines PLHC-1 and RTG-2 was very similar (R = 0.93), although PLHC-1 cells were slightly more sensitive than the RTG-2 cells (Fig. 4B). The reasons behind these slight differences may be related to the higher growth temperature and the faster proliferation (Clemedson *et al.* 1998a; Clemedson *et al.* 1998b) of the PLHC-1 cells (30°C) compared with the RTG-2 cells (21°C) or to a more pronounced metabolism of PLHC-1 cells.

Our results demonstrate that the cytotoxicities of pharmaceuticals in both the NR and MTT assay are basically the same, and therefore, both cytotoxicity assays gave equal results. This is consistent with previous studies, where the EC50-values in the MTT and NR assay were in the same order of magnitude for a series of organotin compounds (Borenfreund *et al.* 1988; Brüschweiler *et al.* 1995), phenols (Fent and Hunn 1996), and an organophosphorus insecticide (Li and Zhang 2002).

The cytotoxicity of some pharmaceuticals has previously been assessed in different cell lines. A comparison of cytotoxicities of chemicals including four pharmaceuticals showed similar sensitivities of mammalian and fish cell lines (Castano et al. (2005)). Our data with caffeine, diazepam, paracetamol and propranolol were also almost identical to the reported data. Laville et al. (2004) determined the cytotoxicity of

fenofibrate, fluoxetine, propranolol, diclofenac, and carbamazepine in PLHC-1 cells and primary cultures of rainbow trout hepatocytes. PLHC-1 cells seemed to be more sensitive than the primary cells. Our data correspond well with these data in case of fluoxetine, propranolol and diclofenac, with the exception of fenofibrate and carbamazepine, where we did not observe cytotoxicity.

Five pharmaceuticals were found to lead to an increase in absorbance at low and non-cytotoxic concentrations in the MTT assay in the PLHC-1 cells. This is interpreted as a hormesis effect, which has already been observed for example after exposure of PLHC-1 cells to cadmium (Ryan and Hightower 1994), and of RTG-2 cells to zinc salts (Ni Shuilleabhain *et al.* 2004). Often, hormesis is associated with an increase in cell proliferation compared to controls (Ni Shuilleabhain *et al.* 2004), but this could not explain why this effect is found in our study with the MTT assay and PLHC-1 cells only. Possibly the uptake of MTT is increased or the dehydrogenase enzyme responsible for the cleavage of the MTT in the mitochondria is increasingly being produced in PLHC-1 cells. The lack of this effect in RTG-2 cells may be related to a lower metabolic activity.

The cytotoxicity of the pharmaceuticals has been found to be related to their LogD-value (Fig. 6). Baseline toxicity is believed to be a result of non-specific disturbance of membrane integrity and functioning as a result of the partitioning of chemical compounds into biological membranes (van Wezel and Opperhuizen 1995). The LogD-value predicts the situation in the cytotoxicity assays more accurate than the K_{OW}-value as it considers the partition of a compound at a specific pH, in our case at physiological conditions with pH 7.0. The EC50-values of the pharmaceuticals showing a correlation with their LogD indicates that the cytotoxic effect is probably due to non-specific toxicity or narcosis. The cytostatic drug doxorubicin and the neuroactive compound fluoxetine exhibited a clearly lower EC50-value than expected from their LogD-value. Also atorvastatin, propranolol and diclofenac deviated from the other compounds. Fluoxetine, propranolol and diclofenac are known to exhibit rather thigh acute and/or chronic toxicity on aquatic organisms (Fent *et al.* 2006). About the statin atorvastatin and the cytostatic doxorubicin only few is known. The high cytotoxic effect of doxorubicin is due to its intercalation in the DNA and RNA thereby interrupting the DNA and RNA synthesis. Furthermore the inhibition of topoisomerase II and the formation of radicals leads to DNA strand breaks (Forth *et al.* 2001), and hence inhibition of cell proliferation. Therefore a specific mode of toxic action probably occurs for five

pharmaceuticals out of 34 assessed in this study. All other substances show a clear correlation with their LogD value indicating cytotoxicity due to non-specific action or narcosis ($R = 0.9805$; $P < 0.0001$).

Our *in vitro* data in fish cell lines were found to correlate to *in vivo* data in *Daphnia* (Fig.7A). In the correlation, pharmaceuticals acting in a non-specific way as well as in a specific way were represented. This suggests that the mechanism of toxicity *in vitro* and *in vivo* is the same. Acute toxicity data from *Daphnia* mainly determined at an exposure time of 48 h were less heterogeneous than data from fish, for which only a sparse set of acute *in vivo* data was found in the literature (Fent *et al.* 2006). Furthermore, the fish data themselves are heterogeneous, showing high variability with species of fish, times of exposure and experimental conditions. Therefore, no correlation can be found between our *in vitro* data in fish cell lines and acute *in vivo* data in fish (Fig. 7B). The fact that a correlation exists with a more homogenous set of data in *Daphnia* suggests that *in vitro* cytotoxicity data may also be correlated with more homogeneous *in vivo* data from fish (if based on similar species and exposure time).

Conclusion

Our results show that fish cell lines are an excellent tool for cytotoxicity assessment of pharmaceuticals. Both the MTT and NR assay are equally well suited for the cytotoxicity assessment. The different cell lines showed cytotoxicity in a similar range, although PLHC-1 cells were slightly more sensitive than the RTG-2 cells. The EC50-values of the compounds were correlated to the partition coefficient LogD of pharmaceuticals indicating that cytotoxicity was prevailing due to unspecific toxicity or narcosis. However, some pharmaceuticals known to exhibit higher toxicities on aquatic organisms deviated from the dataset indicating a specific mode of toxic action.

A correlation is found with acute toxicity data from *Daphnia,* indicating that *in vivo* toxicity may be estimated from *in vitro* experiments in fish cells. The advantages of *in vitro* assays are their simple handling, high reproducibility, and the possibility to screen a large number of compounds in a short period of time. The MTT and NR assays used in this work are well established cytotoxicity assays that have already widely been applied to a broad set of different compounds (Babich and Borenfreund 1987; Babin

and Tarazona 2005; Brüschweiler *et al.* 1995; Castano and Gomez-Lechon 2005; Ni Shuilleabhain *et al.* 2004; Saito *et al.* 1991) and environmental samples (Babin *et al.* 2001; Castano *et al.* 1994; Hollert *et al.* 2000). In the future, cytotoxicity assays in fish cell lines can be a valuable tool in the risk assessment to estimate and rank the acute toxicity of compounds in order to minimize acute toxicity tests *in vivo*. They may be included in a tiered approach to assess the ecotoxicity of pharmaceuticals, but may also gain importance in the toxicity assessment of chemicals in the future strategy of the European Union within the REACH concept. Further investigations are needed with a broader set of compounds and the correlation with *in vivo* data has to be further validated.

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CHAPTER 3

Development and characterization of P-glycoprotein 1 (Pgp1; ABCB1) mediated doxorubicin-resistant PLHC-1 hepatoma fish cell line

Roko Zaja[a], Daniel Caminada[b, c], Jovica Lončar[a], Karl Fent[b, d] and Tvrtko Smital[a]

[a]Laboratory for Molecular Ecotoxicology, Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenicka 54, 10000 Zagreb, Croatia;
[b]University of Applied Sciences Northwestern Switzerland, School of Life Sciences, Institute of Ecopreneurship, Gründenstrasse 40, CH-4132 Muttenz, Switzerland;
[c]University of Zürich, Institute of Plant Biology, Division of Limnology, Seestrasse 187, CH-8802 Kilchberg, Switzerland
[d]Swiss Federal Institute of Technology (ETH), Department of Environmental Sciences, CH-8092 Zürich

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Abstract

The development of the multidrug resistance (MDR) phenotype in mammals is often mediated by the overexpression of the P-glycoprotein1 (Pgp, ABCB1) or multidrug resistance associated protein (MRP)-like ABC transport proteins. A similar phenomenon has also been observed and considered as an important part of the multixenobiotic resistance (MXR) defence system in aquatic organisms. We have recently demonstrated the presence of ABC transporters in the widely used *in vitro* fish model, the PLHC-1 hepatoma cell line. In the present study we were able to select a highly resistant PLHC-1 sub-clone (PLHC-1/dox) by culturing the wild type cells in the presence of 1 μM doxorubicin. Using quantitative PCR a 42-fold higher expression of ABCB1 gene was determined in the PLHC-1/dox cells compared to non-selected wild type cells (PLHC-1/wt). The efflux rates of model fluorescent Pgp1 substrates rhodamine 123 and calcein-AM were 3- to 4-fold higher in the PLHC-1/dox in comparison to the PLHC-1/wt cells. PLHC-1/dox were 45-fold more resistant to doxorubicin cytotoxicity than PLHC-1/wt. Similarly to mammalian cell lines, typical cross-resistance to cytotoxicity of other chemotherapeutics such as daunorubicin, vincristine, vinblastine, etoposide and colchicine, occurred. Furthermore, cyclosporine A, verapamil and PSC833, specific inhibitors of Pgp1 transport activity, completely reversed resistance of PLHC-1/dox cells to all tested drugs, resulting in EC50 values similar to the EC50 values found for PLHC-1/wt. In contrast, MK571, a specific inhibitor of MRP type of efflux transporters, sensitized PLHC-1/dox cells, neither to doxorubicin, nor to any other of the chemotherapeutics used in the study. These data demonstrate for the first time that a specific, Pgp1 mediated doxorubicin resistance mechanism is present in the PLHC-1 fish hepatoma cell line. In addition, the fact that low micromolar concentrations of specific inhibitors may completely reverse a highly expressed doxorubicin resistance points to the fragility of Pgp1 mediated MXR defence mechanism in fish.

***Key words*:** Pgp1, fish cells, MXR, MDR, induction

Introduction

The major obstacle to successful cancer treatment is tumour resistance to a wide spectrum of chemotherapeutics. The development of multidrug resistance (MDR) phenotype is characterized by the marked increase in tumour resistance to structurally different antineoplastic drugs (Ricardo, 2006). The MDR phenotype can arise as a result of different molecular mechanisms, like differential expression of topoisomerase IIα, subcellular redistribution of drug into lysosomes, induction of detoxifying enzymes or alterations of the genes and proteins involved in the control of apoptosis (Ades et al., 2006; Gong et al., 2003; Townsend and Tew1, 2003; Kaufmann and Vaux, 2003). However, one of the major and most studied MDR mechanisms is the enhanced ability of tumour cells to actively efflux drugs, leading to lower concentrations inside the cells and lower toxic potential of chemotherapeutics (Bates, 2003). MDR associated with increased efflux of drugs is a result of overexpression of several members of the ATP-binding cassette (ABC) family of membrane transport proteins. Through binding and hydrolyzation of ATP, ABC proteins obtain the energy necessary for active transport of their substrates across cell membranes. In mammals, the ABC superfamily of proteins is subdivided into seven families designated A through G (http://nutrigene.4t.com/humanabc.htm). Different mammalian models indicate that members from the ABCB1 (MDR, P-glycoprotein), ABCC (multidrug resistance associated proteins, MRPs) and ABCG2 (breast cancer resistance protein, BCRP) family are directly involved in the efflux of xenobiotics and/or their metabolites, representing the toxicologically most relevant ABC transport proteins (Leslie et al., 2005).

Because of its overexpression in various tumour tissues and cell lines the P-glycoprotein 1 (Pgp1) became the first and best characterised ABC transporter. The Pgp1, product of the ABCB1 (MDR1) gene, is a 170 kD glycoprotein organised in two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs). Each of the TMD consists of six alpha-helices which together form two substrate binding sites. The binding of a substrate results in the activation of one ATP-binding domain which hydrolyses ATP. This enables the conformational changes and leads to the extrusion of the substrate out of the cell (Callaghan et al., 2006). Through the described mechanism Pgp1 transports a wide range of structurally diverse xenobiotics, which have common properties such as moderate hydrophobicity, amphiphilicity, neutral or positive charge, a basic nitrogen atom and a high molecular weight (Litman et al., 2001). A vast number

of Pgp1 substrates are important anti-cancer agents, like anthracyclines (doxorubicin, daunorubicin, epirubicin), vinca alkaloids (vinblastine, vincristine), taxanes (paclitaxel, docetaxel), epipodophyllotoxins (etoposide, teniposide), topotecan, actinomycines (actinomycin D), alkylating agents (mytomycin C), peptide antibiotics (valinomycin, gramicidin) and antigout agents (colchicin) (Chan et al., 2004).

The initial observation that many populations of aquatic organisms can survive in a highly polluted environment triggered the first identification of the Pgp-like efflux activity in aquatic organisms (Kurelec and Pivčević, 1989). The related phenomenon was soon termed the MultiXenobiotic Resistance mechanism (MXR; Kurelec, 1992) and the presence and function of Pgp1 (ABCB1) has been identified in almost 40 aquatic organisms investigated so far (Bard, 2000). Similarly to MDR in mammals, Pgp1 catalyzes the efflux of many structurally different xenobiotics out of the cell, reducing their cytotoxicity (Britvić and Kurelec, 1999). The induction of Pgp-like proteins, as important feature of MXR, has been shown at protein and functional levels in different tissues of fish and mussels (Minier et al, 1993; Tutundjian et al., 2002; Smital et al., 2003; Williams et al., 2003). However, it has been demonstrated that specific class of environmental compounds of both, natural or anthropogenic origin, can lead to potent inhibition of Pgp-like proteins (Smital et al, 2004). Through competitive or non-competitive blockage of Pgp1 mediated efflux, those MXR inhibitors or chemosensitizers can cause the increased concentration of toxic compounds inside cells (Smital and Kurelec, 1998).

Besides Pgp1, other proteins from the ABC family, especially MRPs (ABCC) and BCRP (ABCG2), have recently been detected in aquatic organisms (Cai et al., 2003; Sauerborn et al, 2004). Although members of the MRP family predominantly mediate efflux of xenobiotics or their conjugated metabolites (glutathiones, glucoronides or sulphate esters), some of them also transport physiological substrates such as hormones and bile salts (Deeley et al., 2006). Similarly, apart from transporting different xenobiotics (mitoxantrone, etoposide), BCRP (ABCG2) is also implicated in the transport of important physiological molecules like cholesterol, and most recently, its role in the metabolism of hem has been identified (Doyle et al., 2003; Latunde-Dada et al., 2006). Consequently, chemicals that can interact with either Pgp1 or the other efflux transporters like MRPs and BCRP, may not only directly modulate the toxic potential of different xenobiotics, but may also interfere with important physiological

processes. Due to that fact it is important to identify and characterize ABC proteins involved in the overall MXR phenomenon in aquatic organisms, as well as to develop relevant models that could be used in the detection and characterization of MXR inhibitors among conventional and emerging environmental pollutants.

The PLHC-1 hepatoma cell line derived from topminnow (*Poeciliopsis lucida*) is frequently used in aquatic ecotoxicology (Fent, 2001). This cell line is well characterized to the presence of phase I (i.e. CYP1A), and to lesser extent phase II enzymes (GSTs, UGTs) and has been widely used for identification and evaluation of overall toxicity, CYP1A induction and genotoxic potential of individual compounds and complex environmental samples (Hahn et al., 1996; Fent and Bätscher, 2000). Other toxic responses like lipid peroxidation and induction of metallothioneins and heat-shock proteins were also successfully determined using PLHC-1 cells (Rau et al., 2004; Schlenk and Rice, 1998; Babich et al., 2001, Caminada et al., 2006). Most recently, our group described the expression of two (eco)toxicologically relevant ABC transporters, Pgp1 (ABCB1) and MRP3 (ABCC3) in PLHC-1 cells (Žaja et al., 2006). The initial characterization of these two proteins using model fluorescent substrates and model inhibitors revealed similar substrate/inhibitor profiles as those found for mammalian transporters.

In order to better understand the MXR mechanism in fish the aim of the present study was the selection and characterization of highly doxorubicin-resistant fish PLHC-1 cells. Similarly to mammalian multidrug resistance, the obtained resistance cells (PLHC-1/dox), selected by culturing wild cells in high doxorubicin concentrations, exhibited pronounced Pgp1 (ABCB1) expression, resulting in decreased basal accumulation of model fluorescent substrates and cross-resistance to a wide range of chemotherapeutics.

Material and methods

Chemicals

Cyclosporin A (CYC), rhodamine 123 (Rh123), monochlorbimane (MCB), verapamil (VER), vincristine sulfate (VCR), vinblastine sulfate salt (VBL), colchicine (COL), diammineplatinum (II) dichloride (CIS), daunorubicin hydrochloride (DNR), etoposide

(ETO), methotrexate (MET), 1-chloro-2,4-dinitrobenezene (CDNB), buthionine sulfoximine (BSO), N-Acetyl-L-cysteine (NAC), 5,5'dithio-bis-2-nitrobenzoic acid (DNTB), thiazolyl blue tetrazolium bromide (MTT), Triton X-100, dimethyl sulphoxide (DMSO), phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS), pyruvat and HEPES were purchased from Sigma, St. Louis, MO, USA. Doxorubicin hydrochloride (DOX) was obtained from Pharmacia & Upjohn S.p.A. (Milan, Italy). Glutathione was purchased from BDH (Poole, England). Minimum Essential Medium (MEM) and fetal bovine serum (FBS) were obtained from Gibco (Karlsruhe, Germany). MK571 was obtained from Cayman Chemicals Co. (Michigan, OR, USA). Calcein-AM (Ca-AM) was purchased from Molecular Probes (Eugene, OR, USA). *DC* Protein assay kit was obtained from BioRad. Bovine serum albumine (BSA) was obtained from Roth (Karlsruhe, Germany). PSC833 (SDZ 215-833) was kindly provided by Novartis Pharma AG (Basel, Switzerland). Ethanol, isopropanol and all other chemicals used were of the highest analytical grade available and purchased from the Kemika, Zagreb, Croatia.

Growth and selection of PLHC-1 cells

PLHC-1 (*Poeciliopsis lucida* hepatocellular carcinoma) cells were obtained from the American Type Culture Collection (ATCC; LGC Promochem, Teddington, UK). The cells were grown at 30°C in MEM containing Earle's salts, nonessential amino acids, L-glutamine and 5% FBS as previously described (Hahn et al., 1996). Doxorubicin resistant cells were selected by exposure of wild type PLHC-1 cells (PLHC-1/wt) to 1 μM doxorubicin in 75 cm^2 flask (Nunc, Roskilde, Denmark) at 90% cell confluency. After three days of exposure a small number of survived cells were cultured in doxorubicin-free medium and grown up to 70-90% confluence (approximately one month period). The medium was renewed every 72 h. An additional selection was done by repeated exposure of cells to 1 μM doxorubicin for three days. Survived cells were again cultured for seven days to 90% confluence in doxorubicin free medium. Using this procedure, PLHC-1 cells highly resistant to doxorubicin (PLHC-1/dox cells) were selected. From that point the selected cell line was continually cultivated under selection pressure of 0.5 μM doxorubicin. In these conditions PLHC/dox cells exhibited growth rate similar to normal PLHC-1 cells. A part of the PLHC-1/dox cells that were transferred to and cultured in flasks containing doxorubicin-free medium did not show any sign of sensitization during one month period, suggesting that PLHC-1/dox develop a stable resistance phenotype.

Depending on the experimental protocol two days prior to transport activity experiments the cells were seeded in 6-, 48- or 96-wells plates (Tchno Plastic products AG, Trasadingen, Switzerland) in 5, 0.4 and 0.2 mL of doxorubicin free medium per well, resulting in seeding density of 3.5 x 10^5, 3.0 x 10^5 and 1.5 x 10^5 cells per cm^2, respectively.

Real-Time PCR measurements of ABCB1 and ABCC3 gene expression levels

PLHC-1/wt and PLHC-1/dox cells were seeded on a 10 cm^2 cell culture dish and grown to 80-90% confluency in medium with and without 0.5 µM doxorubicin, respectively. The cells were lysed directly in the cell culture dish and homogenized using QIAshredder Kit (Qiagen, Basel, Switzerland). RNA extraction was performed using RNeasy Mini Kit (Qiagen, Basel, Switzerland). The concentration of the RNA was spectrophotometrically measured at 260 nm and the quality was verified on a RNA 6000 Nano LabChip Kit (Agilent Technologies, Basel, Switzerland).

For ABCB1 and ABCC3, the following primers were designed for real time analysis: For ABCB1 Fw 5'-GGAGAAAGCTGGAAAGATCG-3', Rv 5'-AAGGAGAAGGTGAAGCCGTA-3' and for ABCC3 Fw 5'-CAGGAGACAGAGCCAGAAGA-3', Rv 5'-AAGCAAATGATCACCGACAG-3'. β-actin was used as housekeeping gene for normalization. One µg of the total RNA template was reverse transcribed using poly-dT-primer and Transcriptor Reverse Transcriptase (Roche Diagnostics, Basel, Switzerland). Real time PCR amplification was performed on a RotorGeneTM 6000 (Corbett Life Sciences, Brisbane, Australia) using the FastStart SYBR Green System (Roche Diagnostics, Switzerland). The amplification conditions consisted of initial denaturation at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 45 sec, and elongation at 72°C for 45 sec. A melting curve analysis was performed after the run.

The data were analysed using the two standard curve method (Rotor-gene 6000 series software, Corbett Life Science, Australia).

Membrane vesicles preparation and Western blot analysis

The plasma membrane vesicles were prepared from 2 x 10^8 PLHC-1/wt or PLHC-1/dox cells according to the method described by Cornwell et al. (1986). The cells were

scraped, washed once in PBS, resuspended, frozen, thawed, and finally sonicated in 1 ml of vesicle buffer (10 mM Tris, 250 mM sucrose, 0.2 mM CaCl2, 1 mM EDTA, 10 μM PMSF, pH 7.5). The cell lysate was centrifuged (1,000 x *g*, 10 min) and layered on 35% sucrose. After centrifugation at 16,000 x *g* for 30 min in swinging rotor, the layer formed at the top of 35% sucrose was collected, diluted in sample buffer (10 mM Tris, 250 mM sucrose, pH 7.5) and centrifuged at 100,000 x *g* for 1 h. The pellet containing the plasma membrane vesicles was resuspended in 0.5 ml of sample buffer. Total proteins concentration in membrane vesicle fractions was determined according to Lowry et al. (1951). Thirty micrograms of protein per lane were separated by electrophoresis in 7.5% sodium dodecyl sulphate polyacrilamide gel (Laemmli, 1970). The proteins were then transferred to polyvinylidene difluoride membrane by semidry blotting. After blocking and washing steps the membranes were incubated overnight at 4°C with anti-Pgp MAb C-219 (Signet, Dedham, MA, USA). Goat anti-mouse IgG-HRP was used as secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were visualized using Opti-4CN Substrate Kit (Bio-Rad).

Light and fluorescent microscopy

For the purpose of light and fluorescent microscopy experiments, cells were seeded in 24-well microplates at seeding density of 2 x 10^5 cells/cm^2. After 24h, untreated cells were washed in PBS and 0.5 ml PBS was added to each well. Cells were immediately used for light microscopy. For fluorescent microscopy cells were exposed to Ca-AM (0.5 μM) or Rh123 (1 μM) in the presence or absence of CYC (10 μM). After 30 minutes accumulation period cells were washed four times in PBS and finally 0.5 ml PBS was added to each well. To prevent the efflux of fluorescent dyes the plates were kept on ice until microscopic evaluations were performed. Cells were visualised with an inverted fluorescence microscope (Axiovert 40, Zeiss) and CCD camera (Zeiss) using 0.6 second exposure time and same filter set (excitation 450-490, beam spliter 510, emission 515) in the case of both fluorescent dyes. All images were taken with 200x magnification.

Measurements of Pgp1- and MRP-like transport activities

Measurements of Pgp1- and MRP-like mediated transport activities in PLHC-1/wt and PLHC-1/dox cells were performed as previously described, with slight modifications (Žaja et al, 2006). When Rh123 or Ca-AM was used as model substrate the cells were seeded in 48-well culture plates. The cells were washed in PBS, and fresh medium

containing variable concentrations of CYC (dissolved in 96% EtOH), VER (dissolved in 96% EtOH), MK571 (dissolved in DMSO), or PSC833 (dissolved in 96% EtOH) was added to each well. After a short (3-5 min) pre-incubation period with inhibitors Ca-AM or Rh123 (all dissolved in DMSO) were added at desired concentrations in a final volume of 400 µL/well. The final concentrations of the solvents never exceeded 0.1%. The cells were then incubated for 60 min at 30ºC. At the end of incubation period, the cells were washed two times in PBS and finally lysed in 0.1% Triton-X100/PBS (400 µL/well). The fluorescence was measured using microplate reader (Fluorolite 1000, Dynatech, Chantilly, VA, USA) at 485 nm excitation and 530 nm emission wavelengths. The results were expressed as fluorescence units (FU) per $7x10^5$ cells.

When MCB was used as fluorescent substrate, the cells were seeded in 6-well plates 24 h prior to experiments and the retention version of assay was used to measure the MRP-like activity. The cells were washed in PBS and then loaded with 25 µM MCB for 20 minutes at 30ºC. After two washing steps, the inhibitors were added at desired concentrations in 2.5 mL of fresh medium. At the end of the 20 min efflux period, the cells were washed twice in PBS, and finally lysed in 0.1% Triton-X100/PBS (2.5 mL/well). The fluorescence of bimane-GS retained in cells was measured in 96-well plates at 390 nm and 480 nm excitation and emission wavelengths, respectively (Cary Eclipse Microplate reader, Varian Inc., Palo Alto, CA, USA).

Measurements of total glutathione (GSH) levels and glutathione-S-transferase (GST) activity

Cells from one fully confluent 75 cm^2 flask were scraped, centrifuged and finally disrupted by two freezing/thawing cycles in 10 mM Tris-HCl buffer containing 1 mM EDTA (pH=7.4). The obtained homogenates were centrifuged at 10 000 x *g* at +4°C for 10 minutes and the supernatant was used for subsequent measurements. GST activity was determined spectrophotometrically (340 nm) according to Habig et al. (1974). The reaction mixture was composed of 200 µl of assay buffer (100 mM sodium phosphate buffer (pH 6.5), 1 mM GSH and 1 mM CDNB) and 20 µl of sample. The increase in absorbance was monitored during 3 min at 15 sec intervals and specific enzyme activity was calculated and expressed in µmol/min/mg protein.

Total glutathione was measured spectrophotometrically (405 nm) as described by Schlenk and Rice (1998) using DTNB (0.09 mg/ml) as thiol specific reagent. The obtained values were normalised to total proteins and expressed as nmol/mg protein.
Protein concentration in all samples was measured according to the Lowry method (1951) using the BioRad Protein Assay Kit and BSA as standard.

Cytotoxicity assay

Cytotoxicity was determined by the MTT reduction assay adapted according to the Mosmann's procedure (Mosmann, 1983). The cells were seeded in 96-well plates and exposed to a range of concentrations of tested chemicals for 72 h. Subsequently, the medium was removed and the cells were incubated for 3 h with 0.5 mg/ml MTT (50 µl/well) dissolved in MEM. The formazan salts were dissolved in isopropanol and the plates were read on a microplate reader Anthos HT-III (Asys Hitech GmbH, Eugendorf, Austria) at 570 nm using 750 nm as a reference wavelength. Cytotoxicity was expressed as the percentage of product amount formed by mitochondrial activity with respect to the corresponding control (untreated cells) level.

Data analysis

With the exception of gene expression levels in real time PCR experiments (where standard error of means are given), all experimental data are given as mean ± standard deviation and analyzed by t-test and one-way ANOVA (p-value < 0.05). For the purpose of EC50 values calculation the data were normalised and fitted to classical sigmoidal four parameters dose-response model:

$$y = b + (a - b) / (1 + 10^{((LogEC50-x)*h))})$$

where y is the response, *b* represents the minimum of response, *a* represents the maximum of response, *h* is the shape parameter, and x is the logarithm of inhibitor concentration. The *EC50*-value is the concentration of inhibitor that corresponds to 50% of maximal effect. The parameter *a* was constrained to 100 and rest of the parameters were fitted.

The resistance factor (RF) was defined as the ratio of EC50 values obtained with PLHC-1/wt and PLHC-1/dox cells:

$$RF = EC50_{PLHC\text{-}1/dox} / EC50_{PLHC\text{-}1/wt}$$

The potency of inhibitors to reverse the MXR phenotype developed in PLHC-1/dox cells was expressed as the modulation factor (MF) and calculated according to the equation:

$$MF(\%) = (EC50_{PLHC\text{-}1/dox} - EC50\text{-}IN_{PLHC\text{-}1/dox}) / (EC50_{PLHC\text{-}1/dox} - EC50_{PLHC\text{-}1/wt}) \bullet 100$$

where $EC50_{PLHC\text{-}1/dox}$ and $EC50_{PLHC\text{-}1/wt}$ are EC50-values of specific drug obtained for PLHC-1/dox and PLHC-1/wt cells, respectively. $EC50\text{-}IN_{PLHC\text{-}1/dox}$ is the EC50 of drug obtained with PLHC-1/dox cells in the presence of specific transport inhibitor (cytotoxicity modulator).

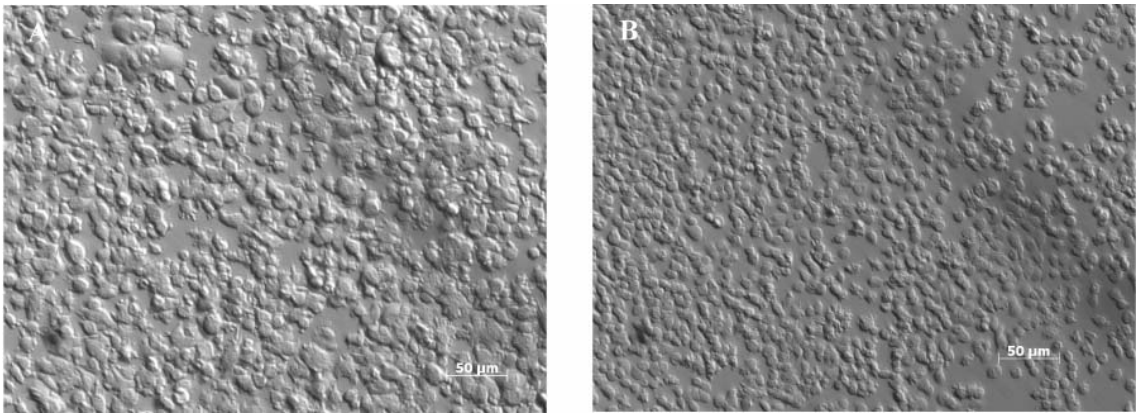
All experiments were independently repeated 3-5 times and the results of typical experiments are shown. All calculations were preformed using GraphPad Prism 4 for Windows and Microsoft Excel.

Results

Morphology of PLHC-1/wt and PLHC-1/dox cells

Both PLHC-1 sub-lines were equally well attached to the surface of cell culture dish and there were no other differences that may be related to different physiology and/or metabolism: growth rate (doubling time) was similar in both sub-lines; nonspecific esterase activities did not reveal any major difference; the total GSH levels were similar; and the activities of crucial phase I (CAP1A as evaluated by the EROD method, data not shown) and phase II (GST) detoxification enzymes were not significantly different. However, marked difference in predominant cell types could be observed between PLHC-1/dox and PLHC-1/wt cells (Fig. 1). PLHC-1/dox sub-line was homogenous consisting predominantly of smaller and round shaped cells. In contrast, PLHC-1/wt sub-line was more heterogeneous consisting mostly of large cells with irregular shape.

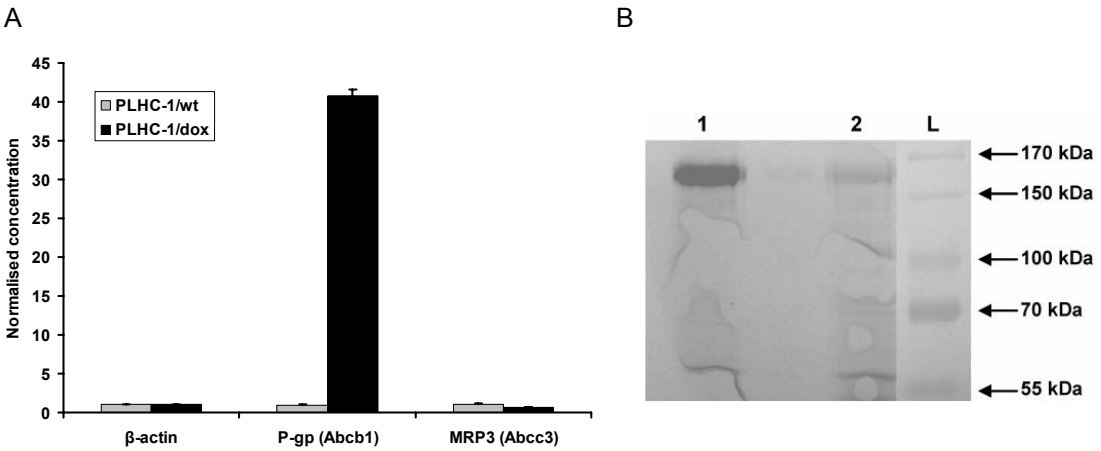
Figure 1. Cell morphology of two PLHC-1 sub-lines. Images of PLHC-1/wt (A) and PLHC-1/dox cells (B) were taken with differential interference contrast (PlasDic, 200 x magnification).



Expression of ABCB1 and ABCC3 in PLHC-1/wt and PLHC-1/dox cells

The expression levels of ABCB1 and ABCC3 were assessed using quantitative RT-PCR (Fig. 2A). The amounts of mRNA for targeted genes were normalized to the mRNA levels of β-actin gene. Relative concentrations of ABCB1 and ABCC3 were similar in PLHC-1/wt cells (0.97 and 1.08, respectively). However, PLHC-1 cells adapted to doxorubicin showed a marked up-regulation of the Pgp1 (ABCB1) gene expression. The relative concentration of ABCB1 in PLHC-1/dox cells was 40.8, which indicates a marked, 42-fold induction when compared to PLHC-1/wt cells. In contrast, the selection of cells with doxorubicin resulted in slight down regulation of ABCC3 gene

Figure 2. Expression of Pgp1 (ABCB1) and MRP3 (ABCC3). (A) Real-Time PCR analysis of gene expression levels. Relative gene expression of Pgp1 (ABCB1) and MRP3 (ABCC3) in PLHC-1/wt and PLHC-1/dox cells were measured and normalized to the gene expression level of β-actin. The values represent means ± standard deviations (n=3). (B) Western blot analysis of Pgp1 (ABCB1) expression in PLHC-1/dox (lane 1) and PLHC-1/wt (lane 2) sub-lines. MAb C-219 was used as the primary antibody. The same amount of total proteins (40 µg/lane) was loaded on gel in case of both, PLHC-1/dox and PLHC-1/wt cells.



– the relative concentration of ABCC3 transcript in PLHC-1/dox cell was 0.64, indicating a 1.7-fold down-regulation in comparison to PLHC-1/wt cells.

Western blot using the MAb C-219 revealed a single band corresponding to a protein of ~165 kDa in membrane fractions of PLHC-1/wt as well as PLHC-1/dox cells (Fig. 2B). However, intensity of this band was markedly stronger in doxorubicin than in wild type PLHC-1 cells, suggesting that over-expression of ABCB1 mRNA is followed by over-expression of Pgp in PLHC-1/dox cells.

Functional characterization of Pgp1- and MRP-like transport activities

Pgp1- and MRP-like transport activities in PLHC-1/wt and PLHC-1/dox cells were measured using three different model fluorescent substrates. Ca-AM and Rh123 are substrates for both Pgp1 and MRP-like transporters (Holló et al., 1996; Daoud et al., 2000). The third substrate used in this study was MCB, a non-fluorescent compound readily taken up by the cells through simple diffusion. Inside the cells it becomes a substrate for glutathione-*S*-transferases which catalyze its conjugation with glutathione (GSH). The resulting bimane-GS conjugate is a highly fluorescent substrate of the MRP subfamily, more specifically of MRP1-4 (Bai et al, 2004; Lou et al., 2003).

Different accumulation rates of CaAM and Rh123 were first demonstrated with fluorescent microscopy. Marked increase in accumulation rates of both dyes were observed in the presence of 10 μM CYC in comparison to wild type cells (Fig. 3). There was also difference in basal accumulations of fluorescent dyes in the two PLHC-1 sub-lines, but the observed discrepancy was more prominent when Rh123 was used as a model substrate (Fig. 3A-B and Fig. 3E-F).

Although the fluorescent microscopy clearly revealed that PLHC-1/wt and PLHC-1/dox cells are different with respect to their transporter activities, in order to obtain more quantitative results and define dose-response relations for other specific Pgp-1 and MRPs inhibitors, accumulation rates of fluorescent substrates were further evaluated using a microplate reader. The basal accumulation of both Ca-AM and Rh123 was approximately three times lower in the PLHC-1/dox cells in comparison to the PLHC-1/wt cells, reflecting higher efflux rates of these fluorescent substrates. In contrast, the retention of MCB-GSH conjugate was higher in the PLHC-1/dox cells (Fig. 4).

Figure 3. Fluorescent microscopy of PLHC-1/wt and PLHC-1/dox cells. Images were taken after 30 min accumulation period in either 0.5 μM calcein-AM (A-D) or rhodamine 123 (E-F), in the absence and presence of 10 μM cyclosporine A. Exposure time was 0.6 s in the case of both fluorescent substrates.

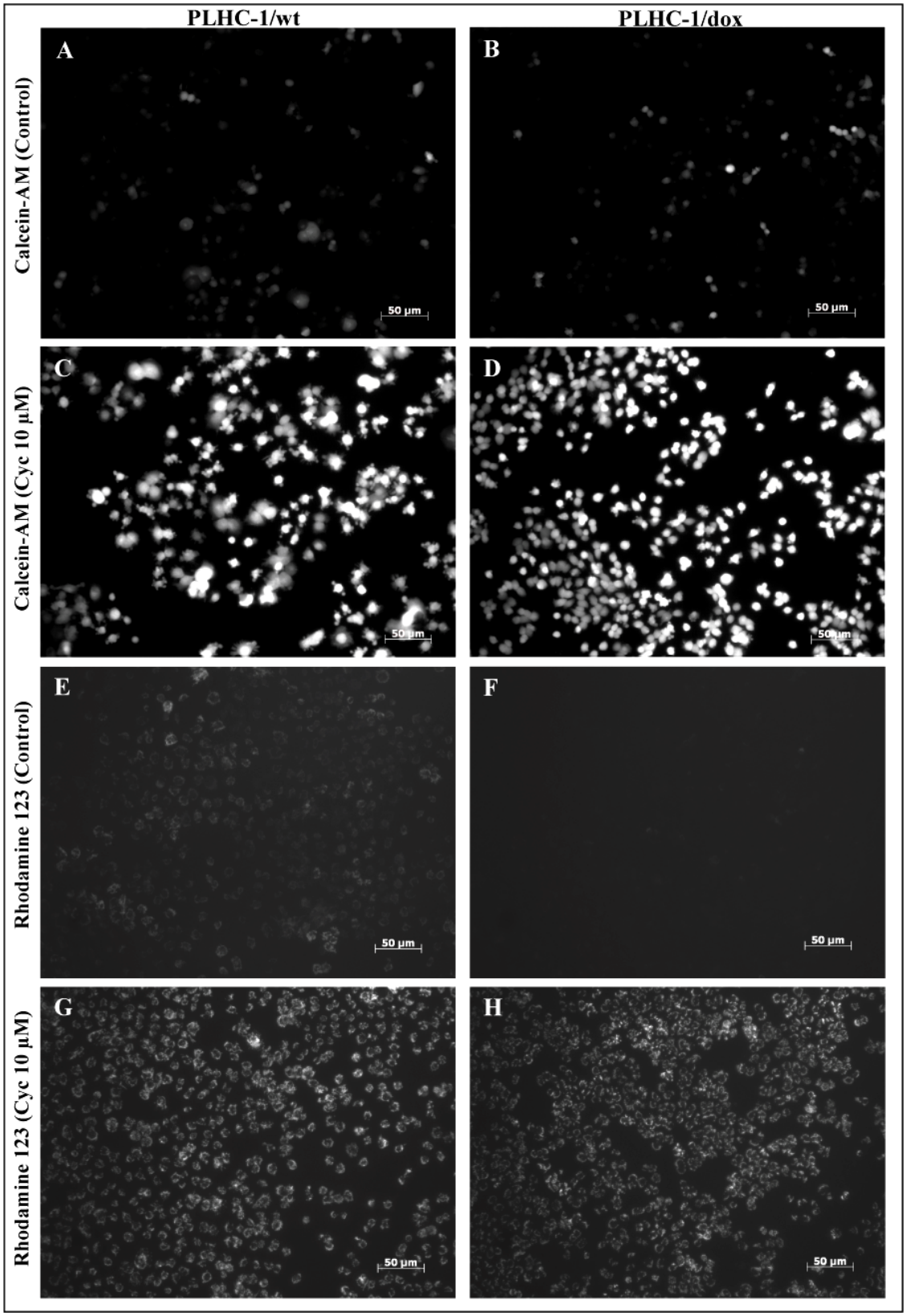


Table 1. EC50 (μM) values and maximal accumulations (fold increase, in parentheses) of fluorescent substrates with model inhibitors used.

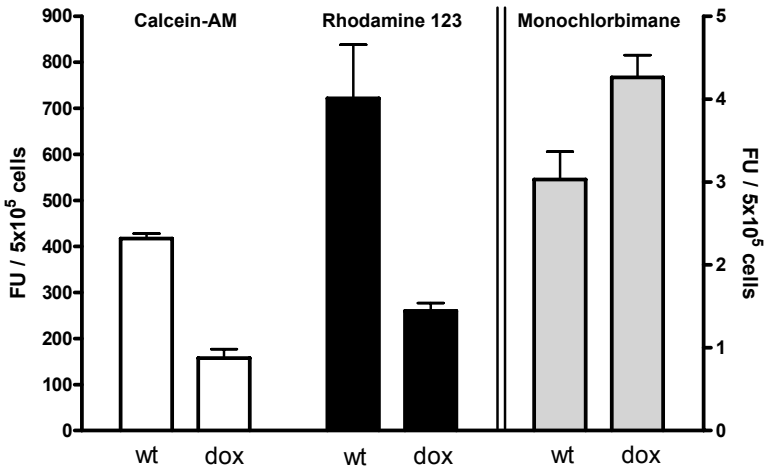
Substrate	Inhibitor	PLHC-1/wt	PLHC-1/dox
Ca-AM	Cyclosporine A	1.7 (2.2)	0.73 (7.1)
	PSC833	0.20 (2.0)	0.53 (7.1)
	Verapamil	*No effect*	6.0 (3.4)
	MK571	1.74 (2.22)	11.4 (2.0)
Rh123	Cyclosporine A	1 (1.6)	0.55 (3.5)
	PSC833	1 (1.4)	0.59 (3.0)
	Verapamil	*No effect*	80 (1.9)*
	MK571	4.1 (3.6)	80 (1.6)*
MCB	Cyclosporine A	3.4 (5.1)	2.9 (2.55)
	MK571	3.5 (4.7)	3.1 (3.23)

* Not fitted values - the numbers represeint maximal accumulation (fold increase) and concentration at which the maximal accumulation was achieved.

The specificity of efflux mechanism was verified using prototypic inhibitors of Pgp1 and MRP-like transporters. The summary of these functional experiments is given in Table 1. In PLHC-1/dox cells all the inhibitors used lead to marked increase in the accumulation of Ca-AM in a dose-response manner. CYC (10 μM) and PSC833 (10 μM) resulted in more than 7-fold increase in Ca-AM accumulation over basal level. Verapamil was less potent and resulted in a 3-fold increase at 20 μM concentration.

The effects of the same inhibitors on Ca-AM accumulation in PLHC-1/wt cells were significantly lower. Although potencies of CYC and PSC833 expressed in EC50 values were similar, the maximal increase in Ca-AM accumulation was only two fold. Similarly,

Figure 4. Basal accumulation rates of model fluorescent substrates. The accumulation levels of calcein-AM (0.25 μM), rhodamine 123 (2.5 μM) and retention of monochlorbimane (25 μM) were measured in the absence of model inhibitors as described in the Materials and Methods section. Data represents means ± standard deviations (n=4) of fluorescent units (FU) determined per $7x10^5$ cells.



no or weak effect on Ca-AM accumulation in PLHC-1/wt cells was observed when VER was used as inhibitor. The opposite effect was observed with MK571, a specific inhibitor of MRP-like proteins. The maximal accumulation of Ca-AM was similar in both cell lines (~2-fold) but a better defined dose-response curve and lower EC50 value was obtained in the PLHC-1/wt cells.

The same pattern of potencies and maximal accumulation was observed with Rh123 as substrate but overall effects were lower than with Ca-AM and well defined dose-response relationships could not be obtained. Again, MK571 exhibited a significantly higher potency in the wild type than in doxorubicin-selected cells. The dose-response curve was well defined yielding an EC50 value of 4.1 µM and a maximal level of Rh123 accumulation of 3.6-fold over the basal level. In PLHC-1/dox, MK571 resulted in low accumulation even at high concentration (80 µM). When MCB was used as substrate, both CYC and MK571 resulted in similar dose-response curves. Estimated EC50 values and maximal accumulations were similar as with PLHC-1/wt cells. Similar results were obtained with PLHC-1/dox cells but lower maximal accumulations were observed for both inhibitors.

Modulation of cytotoxicity

Our Real-Time PCR results and functional experiments clearly indicated that Pgp1 is highly up-regulated in PLHC-1/dox cells. In order to evaluate whether this overexpression would result in a classical MDR phenotype similar to those found in mammalian cells, we determined the cytotoxicity of several commonly used

Table 2. EC50 (µM) values and confidence intervals for tested chemotherapeutics in PLHC-1/wt and PLHC-1/dox cells. The resistance factors (RF) were calculated as simple ratio of EC50 values obtained in the two cell lines.

Drug	PLHC-1/wt	PLHC-1/dox	RF
Doxorubicin	0.144 *0.12 - 0.18*	6.54 *5.06 – 8.46*	**45.3**
Daunorubicin	0.34 *0.29 - 0.40*	3.52 *2.89 - 4.28*	**10.2**
Vincristine	0.0042 *0.0025 - 0.0072*	2.32 *1.50 - 3.58*	**550**
Vinblastine	0.0044 *0.0034 - 0.0056*	0.409 *0.360 - 0.466*	**94.1**
Colchicine	0.13 *0.10 - 0.17*	8.20 *4.98 - 13.51*	**62.2**
Etoposide	4.30 *3.00 - 6.15*	32.80 *26.12 - 41.18*	**7.6**
Methotrexate	0.0024 *0.0017- 0.0033*	0.0016 0.0009 – 0.0028	**0.7**
Cisplatin	19.07 *14.72 - 24.71*	19.58 *19.96 - 29.52*	**0.97**

chemotherapeutics. Obtained EC50 values after 72 h exposure to the tested drugs in both PLHC-1/wt and PLHC-1/dox and the corresponding resistance factors (RF) are summarized in Table 2. The most cytotoxic to PLHC-1/wt cells was MET with EC50 value of 2.4 nM. Two vinca alkaloids, VCR and VBL, exhibited similar toxicity as MET (EC50 values of ~4 nM). Approximately 100-times lower potencies were determined for anthracyclines, DOX and DNR, with respective EC50 values of 144 and 340 nM. The EC50 value obtained for colchicine (130 nM) was in the same range as for the anthracyclines. Etoposide showed approximately ten times lower cytotoxicity, with EC50 value of 4.3 μM. The least potent compound was cisplatin with an EC50 of 19.1 μM.

As can be observed in Fig 5A-F and Table 2, a significant shift in cytotoxicity curves and EC50 values occurred in the PLHC-1/dox cells, demonstrating that PLHC-1/dox cells developed, in addition to doxorubicin, resistance to cytotoxic effects of a range of other compounds, with the exception of cisplatin and methotrexate. The resistance factor (RF) ranged from 7.6, observed for ETO, to more than 550 for VCR (Table 2.).

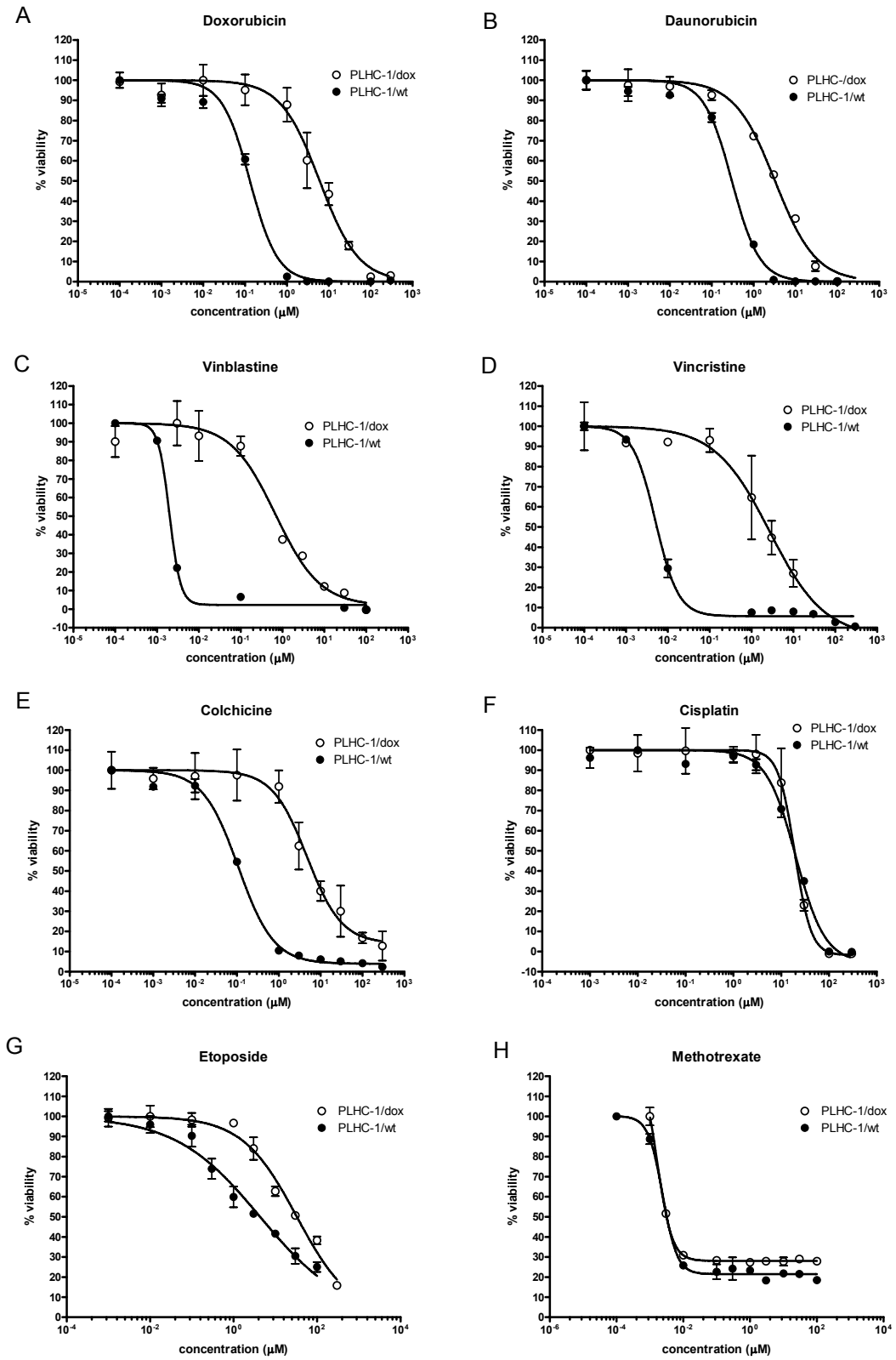
In order to further characterize the underlying mechanism of MDR phenotype observed in PLHC-1/dox cells, the cytotoxicity was measured in the presence of model Pgp1 and MRPs inhibitors. All tested Pgp1 inhibitors lead to almost complete reversion of the MDR phenotype in PLHC-1/dox cells, resulting in EC50 values similar to those determined in wild type cells (Table 3). Low concentrations of CYC (1 μM) and PSC833 (1 μM) resulted in an almost complete (90-100%) reversion of the MDR phenotype against the tested chemotherapeutics. VER was significantly less potent; a

Table 3. Modulator factors (MF) calculated for model inhibitors and cytotoxic drugs tested in two PLHC-1 sub-lines. Data are expressed as percentages

Drug	Verapamil (7.5 μM)	PSC833 (1 μM)	Cyclosporine A (1 μM)	MK571 (7.5 μM)
Doxorubicin	99.7	100.0	99.9	*no effect*
Daunorubicin	91.3	97.2	104.8	*no effect*
Vincristine	83.1	98.6	98.7	*no effect*
Vinblastine	68.5	90.9	100.0	*no effect*
Colchicine	92.9	99.2	99.8	66.5
Etoposide	nt*	nt*	99.5	*no effect*
Methotrexate	*no effect*	*no effect*	*no effect*	*no effect*
Cisplatine	*no effect*	*no effect*	*no effect*	*no effect*

*not tested

Figure 5. Cytotoxicity of chemotherapeutics to PLHC-1/wt and PLHC-1/dox cells. Cells were exposed to a wide concentration range of drugs for 72 h and their cytotoxicity was evaluated using the MTT assay as described in the Materials and Methods section. Data represents means ± standard deviations (n=3).

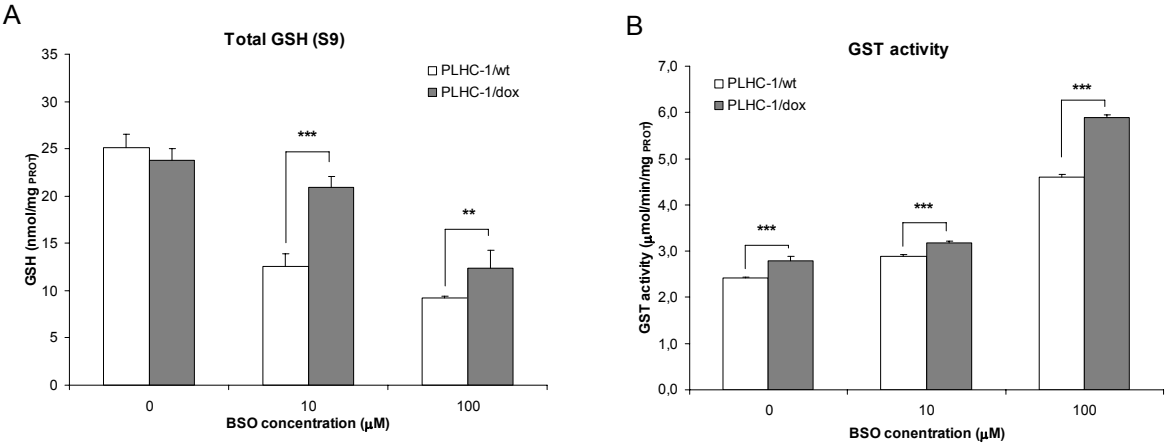


concentration of 7.5 μM did not result in a complete reversion of MDR for all compounds. In the case of VBL, VER resulted in less than 70% of maximal reversion. None of the Pgp1 inhibitors used modulated the cytotoxicity of cisplatin and methotrexate. When MK571 was used as a specific MRP inhibitor a weak cytotoxicity modulation was observed only for colchicine.

Glutathione-S-transferase (GST) activity and total GSH levels

No significant difference was observed between PLHC-1/wt and PLHC-1/dox cells in the levels of total GSH (Fig. 6A). As expected, BSO lead to depletion of GSH level in both cell lines, but PLHC-1/wt cells were more sensitive to BSO exposure. The GST activity was significantly higher in PLHC-1/dox (15%), although the observed increase was probably of no physiological relevance (Fig. 6B). In both cell lines, exposure to BSO resulted in more than two fold induction of GST activity.

Figure 6. Total GSH level and activity of GST. Total levels of GSH (A) and GST activity (B) measured in the absence and presence of BSO for 72 h. Data represents means ± standard deviations (n=4). Asterisks denote statistical significance set at p<0.1 (*), p<0.01 (**) and p<0.001 (***), respectively.



Discussion

MXR is a multifactorial resistance mechanism present in aquatic organisms and mediated predominantly by the activity of ABC efflux transporters. In its nature, this mechanism is similar to well characterized MDR of mammalian cells. The major property of mammalian ABC transporters involved in MDR is their high inducibility. During the last two decades several mammalian cancer cell lines isolated through

selection with different drugs such as doxorubicin, mitoxantrone, etoposide or daunorubicin, exhibited high resistance to a wide range of chemotherapeutics primarily due to the overexpression of Pgp1 (ABCB1), MRP1/2 (ABCC1/2) or BCRP (ABCG2).

In our previous study we demonstrated that fish hepatoma PLHC-1 cells express two ABC efflux transporters possibly implicated in MXR – the Pgp1 (ABCB1) and MRP3 (ABCC3) (Žaja et al., 2006). In the present study we used doxorubicin to select and isolate a PLHC-1 sub-line (PLHC-1/dox) that exhibits a high resistance to structurally different drugs. In order to elucidate the mechanism of resistance found in PLHC-1/dox cells, we measured the gene expression levels of ABCB1 and ABCC3. A pronounced overexpression (> 42-fold) of ABCB1 in PLHC-1/dox in comparison to normal cell line (PLHC-1/wt) was observed (Fig. 1). At the same time, the selection with doxorubicin resulted in slight down-regulation of ABCC3 expression. Although the phenomenon of Pgp1 (ABCB1) overexpression is detected in numerous mammalian cell lines (Bohacova, et al., 2006; Arora et al., 2004), this is the first study proving that a similar mechanism of induction and selection by doxorubicin is present in fish cell lines.

Functional experiments using model fluorescent Pgp1 and MRP substrates confirmed the results of gene expression measurements. PLHC-1/dox cells exhibited 3- to 4-fold lower Ca-AM and Rh123 accumulation rates (Fig. 4). However, in the case of specific MRP1-4 substrate, bimane-GSH conjugate, the retention was slightly higher in PLHC-1/dox cells. Together with gene expression data, functional experiments clearly demonstrated differential regulation of ABCB1 and ABCC3 genes. Doxorubicin acts as a specific and selective pressure leading to selection of PLHC-1 with high and stable overexpression of Pgp1 (ABCB1), while expression of MRP3 (ABCC3) was reduced. Using vesicles obtained from Sf9 cells or cell lines transfected with human MRP3 (ABCC3) several studies showed that doxorubicin is not a substrate of human MRP3 (Zelcer et al., 2001). Consequently, MRP3 could not provide resistance to doxorubicin and no selection of cells expressing high MRP3 levels could be expected. In accordance with these observations, we did not detect higher expression of MRP3 (ABCC3) gene expression in PLHC-1/dox cells.

Almost all of the model Pgp1 and MRPs inhibitors used in this study lead to significant increase in accumulation of prototypic fluorescent substrates in both cell lines. These results are in accordance with our recent study which demonstrates the presence of

Pgp1 and MRP3 in PLHC-1 cells. The PLHC-1/dox cells respond to the model inhibitors in the same way as PLHC-1/wt cells and the maximal accumulation of substrates, in terms of absolute fluorescence per cell achieved in the presence of inhibitor, was approximately the same. The magnitude of inhibitors effect was 2- to 3-fold higher in the PLHC-1/dox cells due to their lower basal accumulation rate. Only the effect of the MRP inhibitor MK571 was approximately the same in PLHC-1/dox as in PLHC-1/wt cells. These data prove the specificity of Pgp1 mediated mechanism underlying the lower basal accumulation of fluorescent substrates in PLHC-1/dox compared to PLHC-1/wt cells.

In mammalian cells, the overexpression of Pgp1 is clearly related to the development of MDR phenotype resulting in high resistance to different chemotherapeutics. In order to evaluate whether the measured overexpression of Pgp1 in fish cells would result in a similar pattern of cross-resistance to a wide spectrum of chemotherapeutics, we performed the cytotoxicity experiments. Among the tested drugs the antifolate methotrexate and two vinca alkaloids, vincristine and vinblastine, were the most cytotoxic to PLHC-1/wt. Approximately hundred times less potent were the antracycline drugs (doxorubicin and daunorubicin). The cytotoxicity of colchicine was in the same range as antracyclines, and the least potent compounds were etoposide and cisplatin. A similar order of cytotoxicity is observed in numerous mammalian cell lines, such as HL60, HepG2, K-562 or MCF-7 (Materna et al., 2005) and in fish cell lines (Caminada et al. 2006). PLHC-1/dox cells exhibited marked resistance to all tested drugs except cisplatin and methotrexate. This indicates that Pgp1 in PLHC-1 fish hepatoma cells posses broad substrate specificity similar to that reported for mammalian cells and tissues. Although the cells were selected with doxorubicin, the highest shift in cytotoxicity was observed for two vinca alkaloids. Relatively high resistance to a structurally unrelated but known substrate of mammalian Pgp1, the antigout agent colchicine, was observed.

Although studies on mammalian cell lines often found high correlation between the increase in expression of efflux transporters (Pgp1 and MRP1/2) and resistance to cisplatin (Nakatani et al., 2005), no resistance of PLHC-1/dox cells towards this compound was measured. However, it is known that resistance to cisplatin is multifactorial and often specific depending on the cell type. Our finding that cisplatin is not a substrate for fish Pgp1 is in accordance with the recent study of Ikuta et al.

(2005), who found that expressions of Pgp1 and Mrp1 are not related to intracellular accumulation of cisplatin, and consequently, that these proteins do not represent a major mechanisms of cisplatin resistance.

Cellular uptake of methotrexate occurs by carrier-mediated transport via reduced folat carrier (RCF1) (Dixon et al., 1994). Once inside cells methotrexate undergoes extensive polygultamylation through activity of folylpolyglutamylate synthetase (FPGS) resulting in highly toxic polyglutamylated metabolites (Moran, 1999). High toxicity of methotrexate indicated that basic components identified in mammalian cells, like RCF1 and FGPS, may also be present in fish cells. It is known that non-polyglutamylated methorexate is substrate of mammalian MRP1-3 (Zeng et al., 2001) and the fact that we did not observe any difference in toxicity between PLHC-1/wt and PLHC-1/dox cells is in accordance with our real-time and functional experiments which showed relatively low expression of MRPs in both PLHC-1 sub-lines.

Further proof of specific Pgp1 mediated resistance of PLHC-1/dox cells towards tested drugs was obtained using specific inhibitors of Pgp1 and MRP-related transport activities. All inhibitors of Pgp1 transport almost completely reversed the resistance of PLHC-1/dox cells to all drugs to which resistance was developed. CYC and PSC833 were the most potent compounds, while a 7.5-times higher concentration of VER was necessary to achieve a similar level of reversal. This order of potencies confirms the findings of functional experiments using fluorescent substrates.

Some of the chemotherapeutics tested in this study are also transported by the members of the MRP family of efflux transporters, but also through activity of BCRP (ABCG2). The only MRP found in PLHC-1 cells is MRP3 (ABCC3) and up to now we were not able to detect gene expression of MRP1 (ABCC1) or MRP2 (ABCC2) in these cells. Our finding that MK571, a potent inhibitor of MRP1-3 did not modulate cytotoxicity of these drugs further demonstrates that MRPs are probably expressed at very low levels in PLHC-1 cells. Furthermore, although etoposide and polyglutamylated forms of methotrexate are also BCRP substrates, novobiocin, a specific inhibitor of BCRP mediated transport, did not result in increased toxicity of these drugs in PLHC-1/dox cells (data not shown). Etoposide is also a known substrate of mammalian Pgp1 and cyclosporine A completely reversed the resistance of PLHC-1/dox cells toward this

compound. These results further suggest that resistance mechanism developed in PLHC-1/dox is highly Pgp1 specific.

The amplification of ABCB1 chromosomal region has been reported as major underlying mechanism of MDR in mammalian drug-resistant cell lines from various cancer types, including breast (Turton et al., 2001), liver (Pang et al., 2005) and ovary (Takano et al., 2001). In addition, selection of cells after point mutation induced by selecting agents, or a combination of point mutation and gene amplification, as proposed by Chen et. al. (1994), may be another possible explanation. Nevertheless, the selection mechanism responsible for doxorubicin resistance in PLHC-1 cells is still not clear. The pictures obtained by light microscopy suggest that PLHC-1/wt cells are a mixture of two cell types. The wild type cells are obviously dominant under normal culture conditions. Yet, under doxorubicin selection pressure a small fraction of cells was able to quickly adapt to doxorubicin, most likely by over-expressing Pgp1. These cells express different morphology and are the predominant type of cells in PLHC-1/dox sub-line. Furthermore, they were able to maintain resistant phenotype even for long period after doxorubicin withdrawal. Recent evidence indicate that small population of tumor cells (~1%) are cells with 'stem like cell' characteristics, and besides high capacity for self-renewal these cells also express high levels of ABC transporters (Lou and Dean, 2007). Consequently, it is possible that exposure to doxorubicin lead to selection of 'stem like cells' with high capacity to quickly over-express Pgp1 and adapt to doxorubicin selection pressure.

Besides, selection with cytotoxic concentrations of MRPs or BCRP substrates in the presence of a specific Pgp1 inhibitor could potentially lead to a development of resistance phenotype mediated through the activity of these transporters. Such experiments, as well as experiments aimed at selection of fish cell lines with other specific types of resistance mechanisms, can further contribute to the knowledge of resistance mechanisms developed in aquatic species.

In conclusion, our study demonstrate that fish PLHC-1/dox cells highly express Pgp1 (ABCB1) mediated multidrug resistance, exhibiting similar cross-resistance pattern to structurally different compounds as the one described in mammalian cells. This finding affirms the PLHC-1 cell line as important model for studying the development of basic resistance mechanisms to different chemicals in aquatic organisms. Furthermore, apart

from being a promising tool for basic and applied research, these data demonstrate relevance of PLHC-1 cells for rapid initial ecotoxicity screening and environmental risk evaluation of drugs. As current techniques rely mainly on *in vivo* systems, the proposed *in vitro* model may help to reduce animal testing in product registration and safety.

Acknowledgments

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CHAPTER 4

Human Pharmaceuticals Affect the Multidrug Resistance Mechanism in the Permanent Fish Cell Line PLHC-1

Daniel Caminada[a, b], Roko Zaja[c], Tvrtko Smital[c] and Karl Fent[a,d]

[a] University of Applied Sciences Northwestern Switzerland, School of Life Sciences, Institute of Ecopreneurship, Gründenstrasse 40, 4132 Muttenz, Switzerland;

[b] University of Zürich, Institute of Plant Biology, Division of Limnology, Seestrasse 187, 8802 Kilchberg, Switzerland

[c] Laboratory for Molecular Ecotoxicology, Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenicka 54, 10000 Zagreb, Croatia;

[d] Swiss Federal Institute of Technology (ETH), Department of Environmental Sciences, 8092 Zürich, Switzerland

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Abstract

The ubiquitous presence of pharmaceuticals in aquatic systems is a challenging problem as their potential effects on aquatic organisms remain largely unknown. The ABC transport proteins contributing to the multidrug/multixenobiotic resistance (MDR/MXR) phenomenon seem to have an important role in the elimination of xenobiotics in aquatic organisms. Modulation of their efflux activities by contaminants may lead to substantial increase in intracellular accumulation and toxic effects of other xenobiotics.

The aim of our work was to analyse a series of pharmaceuticals for their potential to modulate the activity of xenobiotic efflux transporters from the ABCB and ABCC sub-family in the PLHC-1 fish cell line (PLHC-1/wt) and a doxorubicin-resistant PLHC-1 subclone (PLHC-1/dox) characterised by an elevated expression of related ABC transporters. Cellular accumulation of the model fluorescent ABC substrates calcein-AM and rhodamine123 were used to determine an inhibitory effect on efflux transporters. A number of pharmaceuticals had an inhibitory activity with IC50 values occurring in the lower micromolar to millimolar range. Further, cytotoxic effects of pharmaceuticals were analysed in PLHC-1/dox cells. Co-exposure of resistant cells to pharmaceuticals found to have an inhibitory effect and to different pharmaceuticals acting as substrates of ABCB1, resulted in up to five times increased cytotoxicity in comparison to cells exposed without inhibitor.

Our work revealed significant inhibitory effects of environmentally relevant pharmaceuticals on ABC transporters, demonstrating that this class of compounds interacts with the MDR/MXR mechanism in fish. Our findings correspond well with data from mammalian systems indicating that the specificity and roles of the efflux transporters are similarly in both systems. Furthermore, the PLHC-1 cells turned out to be a useful system for the investigation of MDR/MXR mechanisms in fish due to the presence of active and inducible MDR (ABCB) and MRP (ABCC) proteins in this *in vitro* system.

***Key words*:** Multidrug/multixenobiotic resistance (MDR/MXR), pharmaceuticals in the environment, fish cell lines, *in vitro*

Introduction

The ubiquitous presence of pharmaceuticals in aquatic systems has become well documented during the past years (for reviews (Daughton and Ternes 1999; Fent *et al.* 2006; Halling-Sorensen *et al.* 1998)). Various monitoring studies have demonstrated pharmaceuticals and their metabolites from different therapeutic classes in surface waters with median concentrations in the range of ng/L up to µg/L (Calamari *et al.* 2003; Kolpin *et al.* 2002; Wiegel *et al.* 2004). However, only little is known about effects of pharmaceuticals on aquatic organisms and ecosystems, despite their ubiquitous occurrence and high biological activity (Fent *et al.* 2006).

The excretion of xenobiotics through export pumps is often the final step in detoxification processes. In addition, these transporters have been proposed as a first-line defence in aquatic organisms (Epel 1998). In toxicological terms the most important transporters are the P-glycoprotein1 (P-gp1, MDR1, ABCB1), the multidrug-resistance associated proteins MRP1 (ABCC1), MRP2 (ABCC2) and MRP3 (ABCC3), as well as the breast cancer resistance protein BCRP (ABCG2) (Leslie *et al.* 2005). They belong to the ATP-binding cassette (ABC) transporter superfamily and transport a wide range of xenobiotics and metabolites through the cell membrane by hydrolysis of ATP. The consequence is a lower intracellular concentration of xenobiotics and lower toxic potential.

This phenomenon was first observed during cancer treatment and is referred to multidrug resistance (MDR). P-gp1 was found to be the main cause for a resistance towards cytostatic drugs observed during chemotherapy (Juliano and Ling 1976). This 170-kDa membrane protein transports moderately hydrophobic, amphipathic, neutral or positively charged planar organic molecules of low molecular weight with a basic nitrogen atom (Leslie *et al.* 2005). Many anti-cancer agents have been identified as P-gp1 substrates like anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins, topotecan, actinomycines, alkylating agents, peptide antibiotics and antigout agents (Chan *et al.* 2004). Unmodified xenobiotics are mostly transported by P-gp whereas substrates of MRP are direct products of phases I and II metabolism, i.e. they are predominantly present in the form of glutathione, glucuronate or sulphate water-soluble conjugates. Besides xenobiotics, some MRP also transport physiological substrates such as hormones and bile salts (Deeley *et al.* 2006). Many compounds, called chemosensitizers, have been identified that show an inhibitory effect on efflux

transporters and they may have the potential to reverse multidrug resistance (Litman *et al.* 2001; Smital and Kurelec 1998).

In aquatic toxicology, multidrug resistance came into focus when populations of aquatic organisms were observed that were able to survive in highly polluted environments. This phenomenon was soon termed MultiXenobiotic Resistance (MXR) mechanism in contrast to MDR in humans (Kurelec 1992). Indeed, ABCB and ABCC transporters have been found in aquatic organisms such as clams, crabs, mussels, oyster shrimps, snails, sponges, toads, worms and several fish species (Bard 2000). Moreover, an analysis of the zebrafish genome revealed that over 77% of all human ABC transporters have an ortholog in zebrafish (Annilo *et al.* 2006).

The permanent fish cell line PLHC-1 (*Poeciliopsis lucida* hepatoma cell) is regularly used in ecotoxicology as an *in vitro* model system to study detoxification mechanisms (phase I and phase II enzymes) (Fent 2001). The induction of the phase I enzyme CYP1A1 has widely been used for the assessment of the toxic potential of polycyclic aromatic hydrocarbons (PAHs), both as single compounds and as complex environmental mixtures (Fent and Batscher 2000). Furthermore, PLHC-1 cells have been used for the study of heat shock proteins, metallothionein induction and cytotoxicity (Babich and Borenfreund 1991; Caminada *et al.* 2006; Rau *et al.* 2004; Schlenk and Rice 1998). Recently, sequences of the two transporters P-gp1 (ABCB1) and MRP3 (ABCC3) have been detected in the PLHC-1 cell line and the functional characterization of the efflux activity using model fluorescent substrates and model inhibitors revealed similar substrate/inhibitor profiles to those found for mammalian transporters (Zaja *et al.* 2007). In a subsequent work, we succeeded in selecting a doxorubicin-resistant PLHC-1 subclone (PLHC-1/dox) characterized by an about 40-fold overexpression of P-gp1 compared to wild type cells (PLHC-1/wt) (Zaja *et al.* in press). Similarly to mammalian cells, the PLHC-1/dox cells show cross-resistance to cytotoxicity of other chemotherapeutics such as daunorubicin, vincristine, vinblastine, etoposide and colchicine. However, specific inhibitors of P-gp were able to completely reverse the resistance. In contrast to PLHC-1/wt cells, the PLHC-1/dox cells represent a more accurate model system for the specific investigation of effects on P-gp1 and those mediated by P-gp1. Due to the presence of all three critical phases of the detoxification system the PLHC-1 cells (phase I, phase II and efflux transporters) they may serve as a reliable *in vitro* model in aquatic toxicology.

The aim of our present study was to investigate effects of pharmaceuticals found to be present in the aquatic environment on efflux transporters involved in the MDR/MXR mechanism of PLHC-1/wt and PLHC-1/dox cells. Some pharmaceuticals are known to be substrates or inhibitors of mammalian ABCB and ABCC transporters such as statins (atorvastatin, simvastatin), propranolol, tamoxifen and fluoxetine (Peer *et al.* 2004; Ramu *et al.* 1984; Wang *et al.* 2001; Zamora *et al.* 1988), however, data from aquatic organisms for these classes of pharmaceuticals are completely missing. In efflux assays and by fluorescent microscopy, the inhibitory potential of pharmaceuticals was assessed in both subclones using two different fluorescent model substrates. Further, the potential of pharmaceuticals to modulate cytotoxicity in PLHC-1/dox cells was assessed in co-exposure experiments. These studies are important for the interpretation of effects of mixtures of chemicals usually present in the environment, as they may interact by interfering with detoxification systems, thus leading to higher toxicity.

Material and Methods

Chemicals

Dulbecco's minimum essential medium with F-12 nutrient mixture (DMEM/F12) with phenol red, trypsin/ethylenediamine tetraacetic acid (EDTA), and L-glutamine were obtained from LuBio Science GmbH (Switzerland). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich (Switzerland).

Acebutolol hydrochloride (purity >99%), cimetidine (≥ 98%), ciprofloxacin (98%), clofibric acid (97%), cyclosporin A (≥ 98.5%), erythromycin (≥ 95%), fenofibrate (≥ 99%), furosemide (≥ 98%), gemfibrozil (≥ 99%), ibuprofen (≥ 98%), 2,3:4,6,-di-O-isopropylidene-2-keto-gulonic acid monohydrate (≥ 98%), mefenamic acid (> 99%), methotrexate hydrate (> 98%), nonylphenol (techn., 85% of *p*-isomers), norfloxacin (≥ 98%), octylphenol (95%), paracetamol (≥ 99%), (±)-propranolol hydrochloride (≥ 99%), ranitidine hydrochloride (> 99%), rhodamine 123 (≥ 90%), sulfadiazine (99%), sulfamethoxazole (≥ 98%), tamoxifen (≥ 99%), and trimethoprim (98.5%) were purchased from Sigma-Aldrich (Switzerland), calcein-AM from LuBio Sciences GmbH (Switzerland), doxorubicin hydrochloride (≥ 99%), rofecoxib (98.7%), and sildenafil

base (≥ 99%) from Sequoia Research Products Ltd. (United Kingdom), azithromycin (techn.) from Pliva (Croatia), pravastatin (98.7%) from ChemPacific Corporation (USA), and MK571 from ALEXIS Corporation (Switzerland). Bezafibrate (≥ 99.3%) was kindly supplied by F. Hoffmann-La Roche Ltd (Switzerland), and atorvastatin calcium (≥ 99%), diclofenac sodium salt (≥ 99%), fluoxetine (≥ 99%), (±)-metoprolol tartrate (≥ 99%), and simvastatin (98.7%) by Novartis International AG (Switzerland).

Stock solutions of all pharmaceuticals were prepared in DMSO at a concentration corresponding to their solubility. For the different assays, stock solutions were diluted in the cell culture medium resulting in a maximal DMSO concentration not exceeding 2%. Further concentrations were prepared by serial dilution.

Cell culture

The fish hepatoma cells PLHC-1 (*Poeciliopsis lucida* hepatoma cell) kindly supplied by L.E. Hightower (Ryan and Hightower 1994) were grown in DMEM/F12 supplemented with 5% FBS in a humidified incubator with 5% CO_2 at 30°C. Cells were regularly split every 4 days by dissociating with 0.05% (w/v) trypsin and 0.5 mM EDTA and subcultured at split ratios of about 1:6. Wild type and a doxorubicin-resistant subclone of PLHC-1 cells called PLHC-1/wt and PLHC-1/dox, respectively, were used for this study. PLHC-1/dox cells were selected as described before (Zaja *et al.* in press) and are characterized by an increased expression of P-gp1 (ABCB1). As a consequence, they show an elevated resistance to doxorubicin. For the different assays, cells were seeded on 96-well (Huber & Co, Switzerland) or 24-well (Grogg Chemie, Switzerland) tissue culture microtiter plates (MTP).

Measurements of P-gp1 and MRP-like transport activities

Measurements of P-gp1- and MRP-like mediated transport activities in PLHC-1/wt and PLHC-1/dox cells were performed with the model substrates Rhodamine123 (Rho123) or calcein-AM (Ca-AM). 200 µl 80 x 10^4 cells mL^{-1} were seeded in each well of a 96-well tissue culture MTP the day before the assay. For the assay, cells were washed in PBS, and 100 µl fresh medium containing variable concentrations of test compounds or model inhibitors were added to each well. After a short pre-incubation period (5 min) with inhibitors, 100 µl model substrate was added (assay concentration for Ca-AM was 0.25 µM, for Rho123 2.5 µM). The final concentrations of the DMSO solvent never exceeded 2%. The cells were then incubated for 60 min at 30ºC. At the end of the

incubation period, cells were washed twice in PBS and finally lysed in 150 µl 0.1% Triton-X100/PBS. The fluorescence was measured using a microplate reader (Synergene^{TM} 2, BioTek, USA) at 485 nm excitation and 530 nm emission wavelengths. The results are expressed as normalized fluorescent intensity (FI).

Microscopy

For the purpose of fluorescent microscopy experiments, 500 µl of cell suspension was seeded in each well of a 24-well MTP at a seeding density of 80 x 10^4 cells/mL. After 24 h, cells were exposed to 0.25 µM Ca-AM or 2.5 µM Rho123 in the presence or absence of 10 µM cyclosporin A or pharmaceuticals. After 30 minutes accumulation period, cells were washed four times in ice-cold PBS and finally 0.5 ml ice-cold PBS was added to each well. To prevent the efflux of fluorescent dyes the plates were kept on ice until microscopic evaluations were performed. Cells were visualised with an inverted fluorescence microscope (Axiovert 40, Zeiss, Switzerland) and CCD camera (Zeiss, Switzerland) using 300 ms and 500 ms exposure time for Ca-AM and Rho123, respectively, and the same filter set (excitation 450–490 nm, beam spliter 510, emission 515 nm) in the case of both fluorescent dyes. All images were taken with 200-fold magnification.

Cytotoxicity assay

For the cytotoxicity-modulation assays, cells were split and 200 µl cell suspension per well were plated at densities of 20 x 10^4 cells mL^{-1} in 96-well tissue-culture MTP (Huber & Co., Switzerland). After a preincubation of 24 h, PLHC-1/wt and PLHC-1/dox cells were exposed to inhibitors together with substrates of P-gp. Serial dilutions of pharmaceuticals were used as substrates combined with 0.5 µM model inhibitor cyclosporin A, or pharmaceuticals were used as inhibitors along with a serial dilution of the model substrate doxorubicin. The exposed cells were incubated for 72 h. Unexposed cells, cells exposed to the inhibitor and cells exposed to the substrate were used as controls.

MTT Assay. The MTT assay is based on the uptake of methyl thiazolyl blue tetrazolium bromide (MTT) and its following reduction in mitochondria of living cells to MTT formazan, while dead cells are almost completely negative in this cleavage activity (Mosmann 1983). The assay was performed as described previously (Caminada *et al.* 2006). Shortly, cells were washed after 72 h of exposure as described

above with PBS and 220 µl fresh medium was added to each well containing 20 µl MTT solution (5 mg MTT ml^{-1} PBS). The MTPs were incubated for another 4 h before the cell culture medium containing the MTT solution was removed and 200 µl DMSO were added to each well. After shaking for 10 min at 450 rpm, 25 µl Sorensen's Glycine buffer (50 mM glycine, 50 mM sodium chloride/NaOH pH 10.5) was added to each well. Absorption was measured spectrophotometrically at 540 nm on a microplate reader (Synergene™ 2, BioTek, USA). Cell viability was expressed as fraction of negative controls (cells with medium only).

Data analysis

The experiments were performed in three independent experiments in triplicates or quadruplicates and the results of typical experiments are shown. Data were graphically and statistically evaluated with GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA). The raw data were logarithmically transformed.

For the evaluation of the cytotoxicity assays, the data were normalized to a cytotoxic concentration of doxorubicin (positive controls) and to cells grown in medium only (negative control). Wherever applicable, the data were fitted with the four-parameter logistic equation (Hill equation), a nonlinear regression model.

Results

The tested pharmaceuticals were chosen due to their occurrence in the environment and their known potential to interact with human efflux transporters. They act by different modes of action and hence belong to different therapeutic classes including analgesics and anti-inflammatory drugs, blood lipid lowering drugs (fibrates, statins), β-blockers, cytostatic drugs, neuroactive compounds (antiepileptic, selective serotonin reuptake inhibitor), antibiotics and various others (anti-acidic, diuretics).

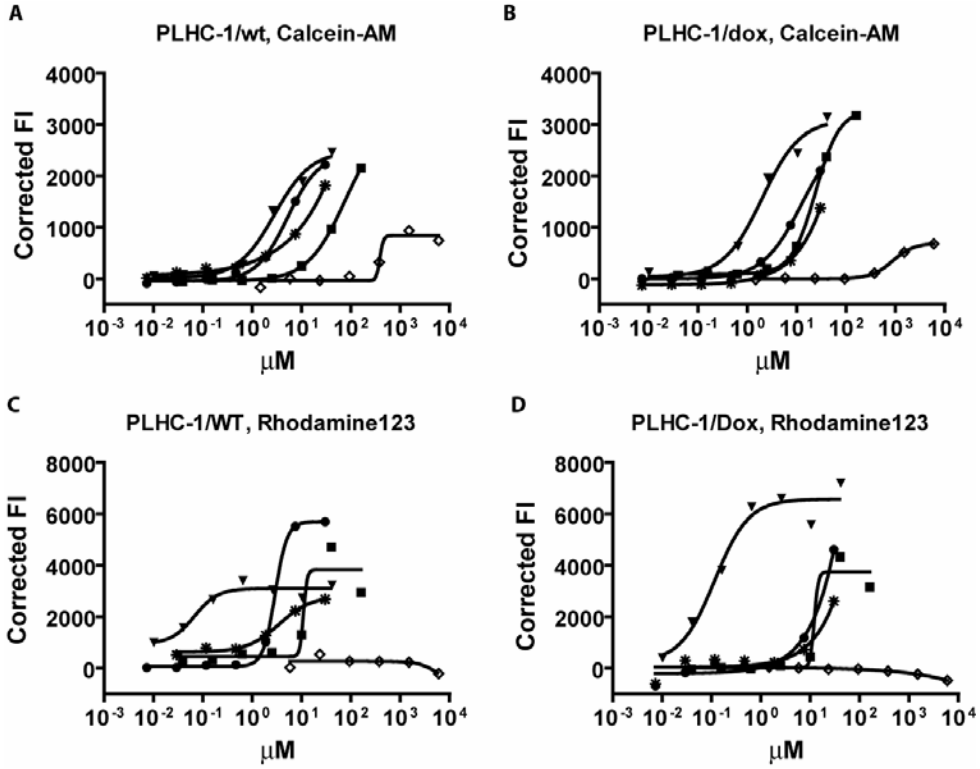
Intracellular accumulation of model substrates in PLHC-1/wt and PLHC-1/dox upon exposition to pharmaceuticals

The potential of pharmaceuticals to inhibit transport activities of efflux transporters was assessed in PLHC-1/wt and PLHC-1/dox cells. Rho123 and Ca-AM were used as fluorescent model substrates. Both have been found to be substrates for P-gp1 and

Table 1: Effects of pharmaceuticals on intracellular accumulation of rho123 and Ca-AM in PLHC-1/wt and PLHC-1/dox cells. Indicated is the x-fold increase in fluorescence of model substrates after 60 min exposition to pharmaceuticals compared to control cells. cMax correlates to the concentration where the highest accumulation has been found. A half-maximal inhibition concentration (IC50) could only be calculated when a full does-response curve was available.

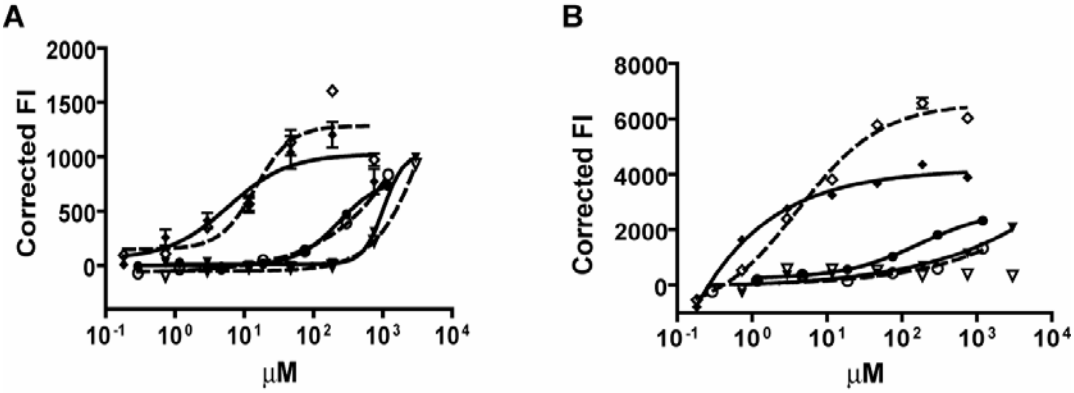
	PLHC-1/WT Rhodamine123			PLHC-1/WT CalceinAM			PLHC-1/DOX Rhodamine123			PLHC-1/DOX CalceinAM		
	x-fold	IC50	cMAX	x-fold	IC50	cMAX	x-fold	IC50	cMAX	x-fold	IC50	cMAX
CyclosporinA	1.68	0.068 µM	0.65 µM	5.83	2.84 µM	40 µM	2.98	0.116 µM	0.65 µM	11.32	2.00 µM	40 µM
MK571	2.14	2.92 µM	7.5 µM	5.34	5.22 µM	30 µM	2.27		30 µM	7.92	12.66 µM	30 µM
Acebutolol	---			---			---			1.34		2500 µM
Atorvastatin	2.1	10.54 µM	40 µM	5.17	78.60 µM	160 µM	2.06	12.09 µM	80 µM	12.87	24.05 µM	160 µM
Bezafibrate	1.7		1500 µM	---			---			1.25		2600 µM
Clofibric acid	1.4		3300 µM	---			---			---		
Diclofenac	1.4		330 µM	---			---			1.14		12 µM
Doxorubicin	---			---			1.12		0.021 µM	1.17		1.3 µM
Fenofibrate	1.1		155 µM	---			---			1.4		155 µM
Furosemide	1.45		3000 µM	3.18		3000 µM	1.18		2.9 µM	3.73		3000 µM
Gemfibrozil	1.5	154.6 µM	1200 µM	2.61	234.8 µM	1200 µM	1.41		1200 µM	3.45		1200 µM
Ibuprofen	1.1		3000 µM	---			---			---		
Mefenamic acid	1.4		100 µM	---			---			---		
Pravastatin	---			2.81	386.6 µM	1500 µM	---			3.53	884.8 µM	6000 µM
Propranolol	---			---			---			2.61	7.98 µM	125 µM
Ranitidine	---			---			---			1.42		2500 µM
Rofecoxib	---			---			---			1.34		225 µM
Sildenafil	2.06	1.37 µM	188 µM	3.63	5.93 µM	47 µM	2.54	4.12 µM	47 µM	4.46	14.86 µM	188 µM
Simvastatin	1.65	3.54 µM	7.5 µM	4.98		30 µM	1.61		30 µM	3.96		30 µM
Tamoxifen	---			---			---			1.41		3.8 µM

Figure 1: Comparison of intracellular accumulation of the model substrates Ca-AM (A and B) and Rho123 (C and D) in PLHC-1/wt (A and C) and PLHC-1/dox (B and D) after exposure to CyA (▼), MK571 (●), atorvastatin (■), simvastatin (✳), and pravastatin (◇).Indicated are means and standard error of means (n=3). The data were normalized to the basal accumulation of model substrates.



MRP-like transporters (Daoud *et al.* 2000; Hollo *et al.* 1996). Effects were found for 18 out of 33 substances evaluated. The antibiotics (ciprofloxacin, erythromycin, 2,3:4,6,-di-O-isopropylidene-2-ketogulonic acid, nonylphenol, norfloxacin, octylphenol, sulfadiazine, sulfamethoxazole, trimethoprim, azithromycin) as well as cimetidine, methotrexate, metoprolol, paracetamol and fluoxetine did not show any effect neither in the PLHC-1/wt nor in the PLHC-1/dox cells. Seven pharmaceuticals (atorvastatin, furosemide, gemfibrozil, sildenafil, and simvastatin) and the two model inhibitors cyclosporin A (CyA) and MK571 showed an increased intracellular accumulation of both model substrates and in both subclones. In general, the difference in accumulation was more pronounced with Ca-AM used as model substrate than with Rho123. Furthermore, the PLHC-1/dox cells exhibited a more pronounced efflux activity due to the higher expression of P-gp. This is apparent in a lower basal accumulation of model substrates (Zaja *et al.* in press). Thus the measured inhibition of efflux of model substrates was more pronounced in the PLHC-1/dox cells. All results are summarized in Table 1.

Figure 2: Intracellular accumulation of calcein-AM (A) and rhodamine 123 (B) in PLHC-1/wt (full symbols) and PLHC-1/dox (open symbols). ▼/▽ furosemide; ●/○ gemfibrozil; ◆/◇ sildenafil. Indicated are means and standard error of means (n=3). The data were normalized to the basal accumulation of model substrates.



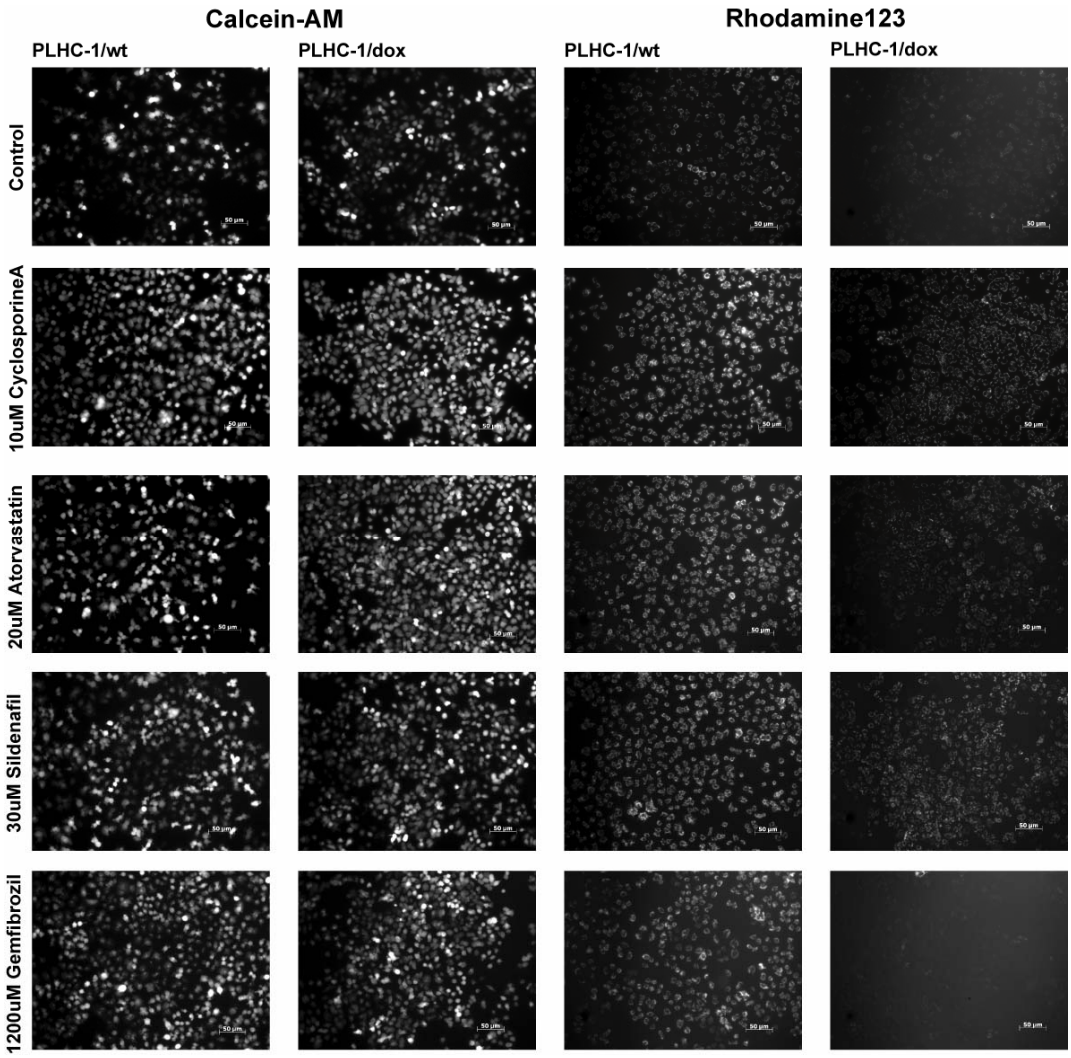
Atorvastatin was the most potent inhibitory pharmaceutical at a concentration of 160 µM. It led to a 5.2-fold and 12.9-fold increase in accumulation of Ca-AM in PLHC-1/wt and PLHC-1/dox cells, respectively. When Rho123 was used as a model substrate, the increase was about 2-fold (Fig. 1 and Tab. 1). These findings are in the same range as the effects found for the model inhibitor CyA. However, atorvastatin was 12-fold less potent as CyA (IC50 with Ca-AM: 24.05 µM for atorvastatin and 2.00 µM for CyA). The further analysed statins simvastatin and pravastatin, yielded an increase in accumulation of up to 5-fold. For pravastatin, no effects were found when Rho123 was used as model substrate, however.

Among the other pharmaceuticals furosemide, gemfibrozil, propranolol, and sildenafil lead to a more than twofold increase of accumulation (Fig. 2). All the other substances had only a slight effect on the efflux activity of the model substrates.

Microscopy

Increased accumulation rates of fluorescent substrates were also observed by fluorescence microscopy when atorvastatin, CyA, furosemide, gemfibrozil, sildenafil, and pravastatin were exposed to PLHC-1/wt or PLHC-1/dox together with the model substrate Rho123 or Ca-AM. These pharmaceuticals lead to a similar accumulation of the fluorescent substrates Ca-AM and Rho123 to the one observed with the model inhibitor CyA. Control cells exposed only to substrate showed clearly lower fluorescence (Fig. 3). There were only slight differences between PLHC-1/wt and PLHC-1/dox cells when Ca-AM was used as a substrate. Exposure to 20 µM atorvastatin led to a higher accumulation of substrate in PLHC-1/dox than in PLHC-1/wt

Figure 3: Accumulation of model substrates calcein-AM (two columns on the left side) and rhodamine 123 (two columns on the right side) observed by fluorescent microscopy. Co-exposure of PLHC-1/wt or PLHC-1/dox cells with model substrate and inhibitor led to increased intracellular accumulation. As inhibitor were used 10 µM CyA, 20 µM atorvastatin, 30 µM sildenafil, 1200 µM gemfibrozil.

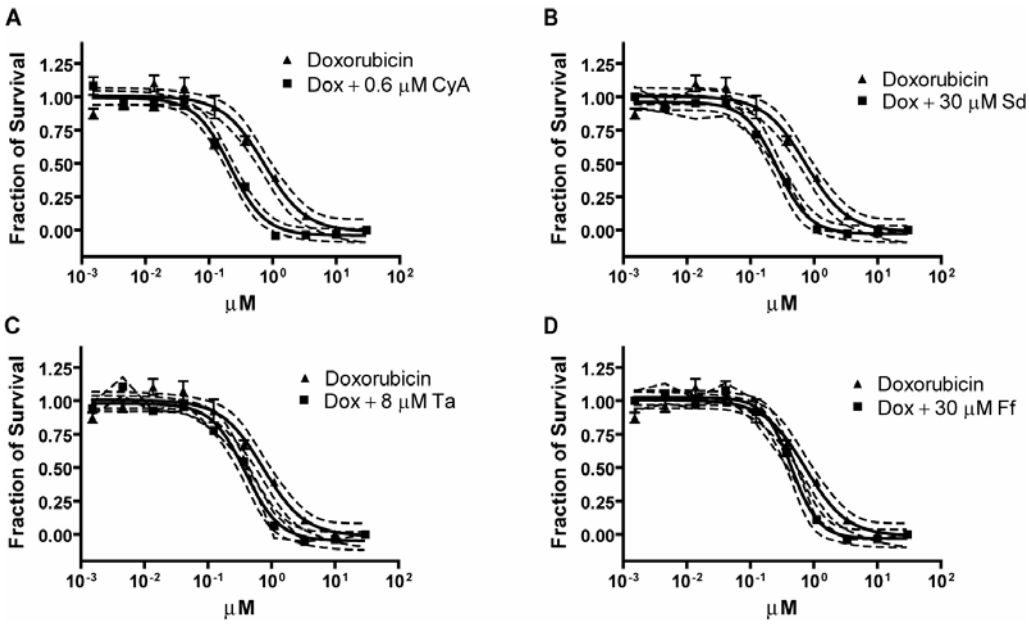


cells, which is in agreement with the findings in the fluorospectrophotometric inhibition assays. When Rho123 was used as substrate, a higher efflux activity was observed with controls and 1200 µM gemfibrozil in the PLHC-1/dox cells indicating that gemfibrozil does not inhibit P-gp like transport mechanisms.

Cytotoxicity

The modulation of efflux activity may lead to higher concentrations of compounds within the cells and therefore to more sensitive responses of the cells. PLHC-1/dox cells export doxorubicin efficiently. Consequently, the EC50 value for cytotoxicity was

Figure 4: Co-exposure experiments performed in PLHC-1/dox cells with doxorubicin as substrate and different inhibitors: A) 0.6 µM CyA, B) 30 µM sildenafil (Sd), C) 8 µM tamoxifen (Ta), D) 30 µM fenofibrate (Ff). The inhibitors modulated the efflux of doxorubicin, which resulted in decreased cytotoxicity compared to cells exposed without inhibitors. The MTT-assay was performed to measure cytotoxicity. Indicated are means, standard error of means and the 95% confidence interval (n=3).



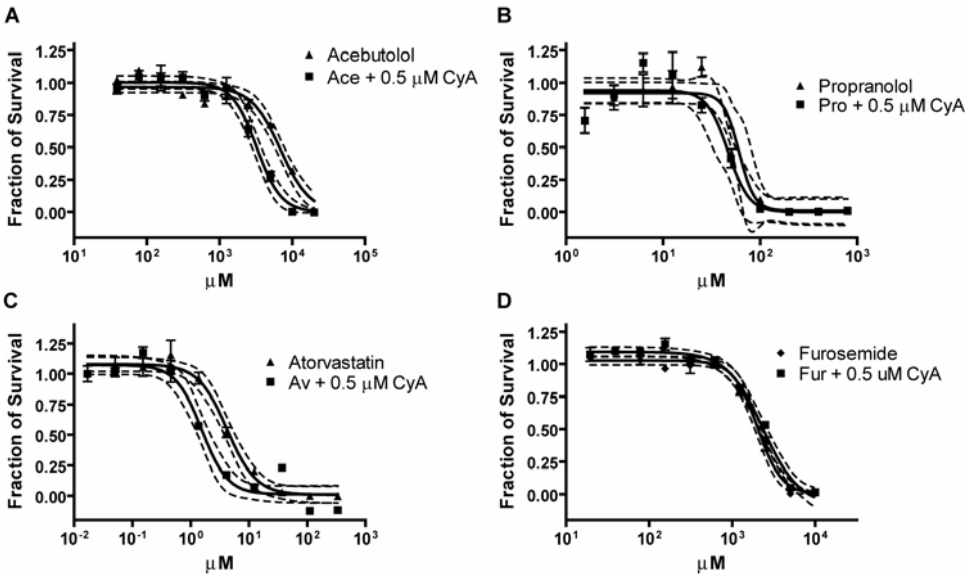
about 5.3 times higher (0.74 µM) than in PLHC-1/wt (0.14 µM) (Tab. 3). Doxorubicin was exposed to PLHC-1/dox and PLHC-1/wt cells together with pharmaceuticals assumed to have an inhibitory effect on P-gp. 8 µM tamoxifen, 30 µM fenofibrate, and 30 µM sildenafil could reverse partly the resistance of the cells towards doxorubicin (Fig. 4). However, atorvastatin, simvastatin and gemfibrozil did not modulate the resistance. This difference was not found in PLHC-1/wt cells (Tab. 2).

Currently, a system sensitive enough to measure ATP hydrolysis is missing for aquatic organisms. This would allow a direct correlation to the transport activity of ABC-

Table 2: EC50 values for doxorubicin found in PLHC-1/dox cells after exposure for 72 h with and without inhibitors. MF: modulation factor (EC50 w/o inhibitor / EC50 with inhibitor).

	EC50 w/o Inhib.	EC50 with Inhib.	MF
0.6 µM CyA	0.740 µM	0.216 µM	3.43
8 µM Tamoxifen	0.740 µM	0.389 µM	1.90
30 µM Fenofibrate	0.740 µM	0.449 µM	1.65
30 µM Sildenafil	0.740 µM	0.264 µM	2.80
15 µM Atorvastatin	0.976 µM	0.723 µM	1.35
10 µM Simvastatin	0.976 µM	1.249 µM	0.78
100 µM Gemfibrozil	0.976 µM	1.224 µM	0.80

Figure 5: Co-exposure experiments performed in PLHC-1/dox cells with 0.5 µM CyA as inhibitor and different pharmaceuticals: A) acebutolol (Ace), B) propranolol (Pro), C) atorvastatin (Av), D) furosemide (Fur). CyA could modulate the cytotoxicity of Ace, Av and slightly Pro indicating that these substances are transported by P-gp. The MTT-assay was performed to measure cytotoxicity. Indicated are means, standard error of means and the 95% confidence interval (n=3).



transporters. However, a shift found in cytotoxicity upon the exposition in PLHC-1/dox cells to an assumed substrate together with an inhibitor gives indirect evidence whether a compound is a substrate of P-gp. Acebutolol, atorvastatin, furosemide, propranolol, and ranitidine pharmaceuticals known to be substrates for human ABCB1 transporters were exposed to PLHC-1/dox and PLHC-1/wt cells together with the model inhibitor CyA. Cytotoxic effects for acebutolol, atorvastatin as well as doxorubicin were found at lower concentrations compared to cells exposed without 0.5 µM CyA (Fig. 5). This was not observed with propranolol and furosemide.

Discussion

In this study we assessed effects of pharmaceuticals from different classes and modes of action in PLHC-1 cells, an *in vitro* model system for fish. PLHC-1 cells were shown to posses MDR activities and the two toxicologically relevant transporters P-gp1 and MRP3 have been cloned (Zaja *et al.* 2007). Furthermore, a P-gp1 overexpressing subclone was selected and characterized towards its functional activity and resistance to various cytostatic drugs. Low concentrations of a specific inhibitor completely

reversed the resistance and revealed the fragility of the P-gp1-mediated MXR defence mechanism in fish (Zaja *et al.* in press).

We screened 33 pharmaceuticals for inhibitory effects on the transport activity using the two model fluorescent substrates Ca-AM and Rho123 both in the PLHC-1/wt and PLHC-1/dox cells. 18 of the 33 tested compounds showed at least in one subclone an increased intracellular accumulation of the model substrates, which demonstrates their inhibitory effects on efflux transporters.

The lipid-lowering agent atorvastatin was the most potent inhibitor and showed a similar inhibitory potency as the model inhibitor CyA on the efflux of both Ca-AM and Rho123. In the cytotoxicity assays, a 2.7-fold shift of the EC50 value to higher toxicity was found in PLHC-1/dox cells when atorvastatin and CyA were co-exposed as compared to solely exposure to atorvastatin. In contrast, atorvastatin could not modulate the cytotoxicity of doxorubicin. These findings indicate that atorvastatin was transported by P-gp and its inhibitory effects were due to competitive inhibition. Simvastatin led to an accumulation of model substrates of up to 5-fold, whereas pravastatin revealed only weaker inhibitory effects and only with Ca-AM as substrate.

In human medicine, statins are used to decrease intracellular cholesterol biosynthesis by reversibly inhibiting the microsomal enzyme hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase (Schachter 2005). However, they have also been found to exhibit effects on MDR transporters. Intracellular accumulation studies with different model substrates and ATP hydrolysis assays were performed to study the characteristics of statins in systems expressing human ABC-transporters. Atorvastatin, simvastatin as well as lovastatin were effective inhibitors of P-gp with IC50 values of 307 µM, 46 µM and 67 µM, respectively (Wang *et al.* 2001). The results of ATP hydrolysis assays in the same study revealed lovastatin and simvastatin as rapid substrates (K_m ~20 µM and ~18 µM for lovastatin and simvastatin, respectively) for P-gp whereas atorvastatin seemed to be a slower substrate (K_m ~84 µM). In contrast, pravastatin did not have any effect on the efflux of Rho123 and an inconclusive effect on ATP hydrolysis. Our studies with fish cells confirm these findings as did other studies on human P-gp (Chen *et al.* 2005; Sakaeda *et al.* 2002).

Sildenafil, the active ingredient of Viagra®, exhibited a 2.1- to 4.5-fold increased intracellular accumulation of model inhibitors and also modulated the cytotoxicity of doxorubicin. In humans, sildenafil was shown to inhibit MRP5 (ABCC5) (Reid *et al.* 2003), an inhibition of P-gp-like transporters has not been reported so far. As the two model substrates Ca-AM and Rho123 are known to be transported by both P-gp and MRP-like transporters we propose that the effect found is at least partly due to the inhibition of a so far not described ABCC transporter in PLHC-1 cells, possibly MRP5. Furthermore, doxorubicin induced not only the expression of P-gp but also of MRP5 in human lung cancer cells (Yoshida *et al.* 2001). The authors proposed that this co-overexpression may contribute to the doxorubicin-resistance found in cancer cells. These findings can explain why sildenafil could modulate the cytotoxicity of doxorubicin. Further studies are necessary in PLHC-1 cells to prove this conclusion.

Weak effects on the transport activity of model substrates were also found for the fibrates gemfibrozil, fenofibrate, bezafibrate and the active metabolite of several fibrates, clofibric acid. 1500 µM bezafibrate and 3300 µM clofibric acid exhibited maximal inhibitory effects of 1.7-fold and 1.4-fold, respectively. The high concentrations and small effects found for bezafibrate and clofibric acid may be explained as unspecific inhibitions of the transporters. In contrast, gemfibrozil showed up to 3.5-fold inhibitory effects with PLHC-1/wt and PLHC-1/dox cells as well as with both model substrates. Fenofibrate inhibited the transport activity of Ca-AM in PLHC-1/dox only slightly but it modulated the cytotoxicity of doxorubicin.

Fenofibrate was found to inhibit moderately human P-gp, whereas gemfibrozil, bezafibrate and clofibric acid did not exhibit any effect on P-gp (Yamazaki *et al.* 2005). However, gemfibrozil showed inhibition on human organic anion transporting-polypeptide 1B1 (OATP2) (Yamazaki *et al.* 2005). Our findings suggest that the effect found for gemfibrozil is not due to an effect on P-gp as the dose-response curve was well defined in PLHC-1/wt but not in PLHC-1/dox cells. Rather, gemfibrozil may interact with other transporters expressed in PLHC-1 cells.

Tamoxifen is used in the treatment against breast carcinoma. It exhibits its antiestrogenic properties due to its ability to compete with estrogen for its binding sites in target tissues (Furr and Jordan 1984). Different studies have demonstrated an inhibitory effect of tamoxifen on human P-gp *in vitro* as well as *in vivo* (Bekaii-Saab *et*

al. 2004; Frank *et al.* 2001; Keen *et al.* 1994; Saeki *et al.* 2005). Our study verified that tamoxifen modulates the cytotoxicity of doxorubicin also in fish cells. In intracellular accumulation assays a 1.4-fold maximal inhibition of tamoxifen occurred at 3.8 µM which is consistent with published IC50 values in the range of 1–30 µM.

The two β-blockers acebutolol and proranolol were analysed in this study as they were reported to be substrates of human P-gp (Kawazu *et al.* 2006; Yang *et al.* 2000). In PLHC-1/dox cells, propranolol led to a 2.61-fold accumulation of Ca-AM, but acebutolol showed only a slight effect. When they were co-exposed with CyA, a shift in cytotoxicity was observed for acebutolol. For propranolol, there was only a small, but not significant modulation. These findings indicate that both β-blockers are moderate substrates of P-gp, however, the sensitivity is not high enough to draw firm conclusion.

In conclusion, our results confirm that the two subclones PLHC-1/wt and PLHC-1/dox are reliable tools for assessing inhibitory effects of human pharmaceuticals to fish ABC-transporters. Cytotoxicity assays showed that inhibitors of multidrug resistance transporters like P-gp can exhibit indirect effects in cells. Furthermore, the modulation of cytotoxicity of pharmaceuticals such as atorvastatin and acebutolol indicate that these substances are transported by P-gp. The findings correlate well with effects found in human systems confirming that the transporters involved in MXR in fish play a similar role as in mammals. For further characterisation of MXR mechanisms in fish it is necessary to clone, transfect and express transporters like P-gp1 and MRP3 in appropriate model systems. However, the PLHC-1 cells are a reliable system for the screening and assessment of single compounds and likely also of environmental samples regarding their effects on MXR in aquatic organisms. Our study also demonstrates that human pharmaceuticals present in aquatic systems interact with efflux transporters, although at much higher concentrations than found in the environment. The interaction leads to inhibition of efflux activity and therefore mixtures of such compounds may increase the toxicity to environmental pollutants.

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CHAPTER 5

Detection of Three Peroxisome Proliferator-Activated Receptors (PPARs) in the Fish Cell Line PLHC-1 and Preliminary Characterisation of Effects after Exposure to Fibrates

Daniel Caminada[1, 2], Anna Weston[1], Karl Fent[1, 3]

[1]University of Applied Sciences Northwestern Switzerland, School of Life Sciences, Institute of Ecopreneurship, Gründenstrasse 40, 4132 Muttenz, Switzerland;
[2]University of Zürich, Institute of Plant Biology, Division of Limnology, Seestrasse 187, 8802 Kilchberg, Switzerland
[3]Swiss Federal Institute of Technology (ETH), Department of Environmental Sciences, 8092 Zürich, Switzerland

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Abstract

Lipid-lowering agents such as bezafibrate, fenofibrate, gemfibrozil and the metabolite clofibric acid belong to those pharmaceuticals most often found in aquatic systems, with concentrations as high as several micrograms per litre. However, only sparse data are available on potential adverse effects in aquatic organisms. The peroxisome proliferator-activated receptor α (PPARα) is the specific targets of fibrates in humans and there is evidence that the latter also interact with these receptors in fish. Upon activation PPARs form a heterodimer with the retinoid X receptor (RxR) and bind to specific regulatory regions in target genes.

The aim of our work was the detection and characterisation of the three receptors PPARα, PPARβ and PPARγ in the fish cell line PLHC-1. By using degenerated primers designed for conserved regions of the different receptors, we found all PPARs as well as RxR present in the cells. The gene sequences showed high similarities to other known sequences in fish, especially with *Dicentrarchus labrax* and *Sparus aurata*. In expression analysis, the highest expression level was found for PPARα followed by PPARγ and PPARβ. Upon exposition to different fibrates for 6 h, the expression level of PPARα and PPARγ was induced up to tenfold. Further, we measured an increased activity of the peroxisomal enzyme fatty acyl coenzyme A oxidase (FAO) after exposure of PLHC-1 cells to fibrates. All fibrates investigated showed effects on FAO, and gemfibrozil showed the highest increase (30%) in FAO activity over control level. These results demonstrate for the first time the presence and function of PPARs in the fish cell line PLHC-1.

***Key Words*:** Fibrates, peroxisome proliferator-activated receptors, peroxisome proliferation, PLHC-1, fish cell line, *in vitro*

Introduction

Pharmaceuticals have frequently been detected in their parent form or as metabolites in effluents of sewage treatment plants, surface waters, ground waters and even in the sea (for reviews (Daughton and Ternes 1999; Fent *et al.* 2006; Halling-Sorensen *et al.* 1998)). Several monitoring studies have shown that many pharmaceuticals from different therapeutic classes are present in surface waters with median concentrations in the range of ng/L up to µg/L (Fent *et al.* 2006). At present, little is known about their potential adverse effects on aquatic organisms and ecosystems, despite their ubiquitous occurrence and high biological activity (Fent *et al.* 2006).

Fibrates and their metabolites belong to pharmaceuticals regularly found in aquatic systems with concentrations as high as several micrograms per litre (Fent *et al.* 2006). Clofibric acid, the persistent and active metabolite of clofibrate, etofyllin clofibrate and etofibrate, belongs to the most frequently detected pharmaceuticals in monitoring studies. It has been found in numerous wastewaters, surface waters, in seawater (Weigel *et al.* 2002) and even in concentrations up to 4 µg/L in the groundwater and in the drinking water (0.07-0.27 µg/L) (Heberer 2002). Bezafibrate, fenofibrate and gemfibrozil have been detected in sewage water and surface water with concentrations up to 4.6 µg/L and 3.1 µg/L, respectively.

Fibrates as another group of pharmaceuticals, the statins, are lipid-lowering drugs. They are used for the treatment of hypercholesterolemia and decrease the concentration of cholesterol – and fibrates also of triglycerides – in the blood plasma. Whereas the statins inhibit the 3-hydoxymethylglutaril coenzyme A reductase (HMG-CoA) (Endo 1992), the fibrates exhibit their effects through alterations in the transcription of genes encoding for proteins controlling the lipoprotein metabolism. The target of fibrates was found to be the peroxisome proliferator-activated receptors (PPAR), manly the PPARα isoform (Staels *et al.* 1998).

PPARs belong to the steroid/thyroid/retinoid receptor superfamily of ligand-activated nuclear transcription factors. Upon activation, they form a heterodimer with the retinoid X receptor (RxR) and bind to specific regions – the peroxisome proliferator response elements (PPRE) – of target genes. To date, three subtypes of PPAR have been described, called PPARα, PPARβ (or PPARδ) and PPARγ. They play a key role in the

lipid metabolism and the lipid homeostasis (Desvergne *et al.* 2006; Escher and Wahli 2000). In addition, all PPAR isoformes can participate in the regulation of inflammatory responses (Escher and Wahli 2000).

Several studies have investigated PPARs in aquatic organisms. Full PPAR coding sequences have been described in fish such as plaice *Pleuronctes platessa* and gilthead sea bream *Sparus aurata* (Leaver *et al.* 1998), seabass *Dicentrarchus labrax* (Boukouvala *et al.* 2004), zebrafish *Danio rerio* (Robinson-Rechavi *et al.* 2001), thicklipped grey mullet *Chelon labrosus* (Raingeard *et al.* 2006), and torafugu pufferfish *Takifugu rubripes* (Kondo *et al.* 2007). In addition, partial nucleotide sequences have been published for atlantic salmon *Salmo salar* (Ruyter *et al.* 1997), rainbow trout *Oncorhynchus mykiss* (Liu *et al.* 2005), brown trout *Salmo trutta* (Batista-Pinto *et al.* 2005), and goldfish *Carassius auratus* (Mimeault *et al.* 2006). Fish PPARs display an amino acid sequence identity of 46–73% to the human and amphibian PPARs (Kondo *et al.* 2007).

Like in humans PPARα was mainly expressed in hepatocyte and tissues that catabolize high amounts of fatty acids (Ibabe *et al.* 2002). Furthermore, PPARγ was shown to be induced in response to clofibrate and bezafibrate in salmon hepatocytes (Ruyter *et al.* 1997), although their PPARγ seem to be less responsive than PPARγ of rodents (Andersen *et al.* 2000). All three PPAR receptors were found to be already expressed in the larval stage, with a similar tissue distribution pattern to that found in adult zebrafish (Ibabe *et al.* 2005a). Activators of PPARα include a variety of endogenously present fatty acids, leukotrienes, hydroxyeicosatetraenoic acids and drugs, such as fibrates (Cajaraville *et al.* 2003). PPARβ activators include fatty acids, prostaglandin A2 and prostacyclin. PPARγ is the most selective receptor and prostaglandin J2 has been described to be a specific ligand (Ibabe *et al.* 2005b). In isolated zebrafish hepatocytes, mRNA of both PPARα and PPARγ was induced by clofibrate at 0.5–2 mM, although to a low extent (Ibabe *et al.* 2005b). The physiological and toxicological roles of PPARs have yet to be investigated, but there is evidence that there may be differences to other vertebrates. Kondo et al. (2007) found that activators of mammalian PPARβ and PPARγ failed to have an effect on these receptors in *Takifugu rubripes*. These finding indicate that the PPARs of *T. rupripes* require either undefined ligands or that the molecular mechanisms involved in their activation are different from those of other vertebrates.

The PLHC-1 cell line (*Poeciliopsis lucida* hepatoma cell) has widely been used in aquatic ecotoxicology as an *in vitro* model system (Fent 2001). The presence of phase I enzymes, especially CYP1A1, and to a lesser extent phase II enzymes (GSTs, UGTs) has been reported. Recently, the expression of two toxicologically relevant ABC transporters, Pgp1 (ABCB1) and MRP3 (ABCC3) has been described (Zaja *et al.* 2007). Furthermore this cell line has been widely used for the identification and evaluation of cytotoxicity, CYP1A induction, genotoxic potential of individual compounds and complex environmental samples, toxic responses like lipid peroxidation, induction of metallothioneins and heatshock proteins (Caminada *et al.* 2006; Fent and Batscher 2000; Rau *et al.* 2004; Schlenk and Rice 1998).

The aim of our study was the detection and primary characterisation of the three receptors PPARα, PPARβ and PPARγ in the fish hepatoma cell line PLHC-1. The receptor sequences found revealed high similarities to other known fish PPARs. In addition, the retinoid X receptor (RxR) was detected, a necessary preliminary for a functional activation by the PPARs. Further, the induction of the receptors was investigated after exposure to fibrates as well as the activity of the fatty acyl-CoA oxidase (FAO) to prove the functionality of the PPAR activation and to assess effects of ecotoxicologically relevant fibrates in the PLHC-1 cell line following a mechanism-based approach to assess ecotoxicity of environmental pharmaceuticals.

Materials and Methods

Chemicals

Dulbeccos minimum essential medium with F-12 nutrient mixture (DMEM/F12) with phenol red, trypsin/ethylenediamine tetraacetic acid (EDTA), and L-glutamine were obtained from LuBio Science GmbH (Switzerland). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich (Switzerland). Dimethyl sulfoxide (DMSO), di-chloro-di-hydro-fluorescamine, aminotriazole, bovine serum albumin (BSA), tween, palmitoyl CoA, clofibric acid (purity 97%), fenofibrate (≥ 99%), and gemfibrozil (≥ 99%) were purchased from Sigma-Aldrich (Switzerland), bezafibrate (≥ 99.3%) was kindly supplied by F. Hoffmann-La Roche Ltd (Switzerland).

Stock solutions of all pharmaceuticals were prepared in DMSO at a concentration of 500 mM where soluble, otherwise substances were diluted corresponding to their solubility. For the different assays, stock solutions were diluted in the buffered cell culture medium (20 mM HEPES/pH 7.2) resulting in a maximal DMSO concentration of 0.05%. Further concentrations were prepared by serial dilution.

Cell culture

The fish hepatoma cells PLHC-1 (*Poeciliopsis lucida* hepatoma cell) kindly supplied by L.E. Hightower (Ryan 1994) were grown in DMEM/F12 supplemented with 5% FBS in a humidified incubator with 5% CO_2 at 30°C. Cells were usually split every 4 days by dissociating with 0.05% (w/v) trypsin and 0.5 mM EDTA and subcultured at split ratios of about 1:6. For the different assays, cells were seeded on cell culture dishes with 6 cm or 10 cm diameter (Milian, Switzerland).

RT-PCR

The PLHC-1 cells were lysed directly on the cell culture dish and homogenized using a QIAshredder Kit (Qiagen, Basel, Switzerland). RNA extraction was performed using an RNeasy Mini Kit (Qiagen, Basel, Switzerland). The concentration of the RNA was spectrophotometrically measured at 260 nm (NanoDrop® ND-1000 full-spectrum (220–750 nm) spectrophotometer) and the quality was verified by measuring the ratio of absorption at 260 nm and 280 nm as well as on a RNA 6000 Nano LabChip Kit (Agilent Technologies, Basel, Switzerland). 1000 ng of the total RNA template was reverse-transcribed using poly-dT-primer and Transcriptor Reverse Transcriptase (Roche Diagnostics, Basel, Switzerland).

For the detection of PPARα, PPARβ, PPARγ and RxR, known sequences of all four receptors were aligned and degenerated primers were designed on conserved regions (Tab. 1). The amplification conditions consisted of initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing for 60 sec, and elongation at 72°C for 60 sec. The final elongation was hold for 7 min at 72°C. A temperature gradient was performed in the range from 45°C to 60°C in order to find the best annealing temperature (Table 1).

The PCR samples were loaded on a DNA 1000 LabChip (Agilent Technologies, Basel, Switzerland) and analyzed on an Agilent 2100 bioanalyzer (Agilent Technologies,

Table 1. Primers designed for the detection of PPARα, PPARβ, PPARγ and RxR. Degenerated positions in the primer sequence are indicated in bold. An_{opt}: optimal annealing temperature. IUPAC-code: R=A/G, Y=C/T, S=C/G, D=A/G/T, B=C/G/T.

Primer	Sequence	Size [bp]	An_{opt} [°C]
PPARα_FW1	5'-ACGGTCAC**R**GAGCT**R**AC**S**GAG-3'	230	55.0
PPARα_RV1	5'-AA**Y**TTGGGCTCCATCATGTC-3'		
PPARα_FW2	5'-GCCAT**Y**ATCTGCTGTGGAG-3'	219	52.0
PPARα_RV2	5'-CG**Y**GTCTTC**Y**GTCTT**S**TTGA-3'		
PPARα_FW1 & PPARα_RV2		513	50.0
PPARβ_FW1	5'-CCTACCTGAAGAACCTCA**D**TATGACC-3'	229	55.0
PPARβ_RV1	5'-GTGAGCTCTCGCAC**B**GTCT-3'		
PPARβ_FW2	5'-TGCGAGAGCTCACCGAGT-3'	448	55.0
PPARβ_RV2	5'-AGGTCGGCCATCTT**Y**TGC-3'		
PPARβ_FW1 & PPARβ_RV2		664	55.0
PPARγ_FW1	5'-TGTGATCTTCACTGTCG**S**ATTC-3'	323	55.0
PPARγ_RV1	5'-AGAGACTTCATGTCATGGATGA-3'		
RxR_FW1	5'-ACTGCCGCTACCAGAAGTGT-3'	555	58.0
RxR_RV1	5'-ACCCCAGTTCTGTCTTATCCAT-3'		

Basel, Switzerland). For sequencing, PCR samples were loaded and separated on a 1.2% agarose gel and the bands of expected sizes were excised from the gel. The following digestion of the agarose pieces and the purification of the PCR products were performed using NucleoSpin® Extract II kit (Macherey-Nagel, Germany). The PCR products were custom-sequenced (Synergene Biotech GmbH, Switzerland).

Expression analysis of PPARs

PLHC-1 cells were exposed for 6 h to different fibrates. The cells were seeded on cell culture dishes (6 cm diameter) with a density of $10x10^5$ cells/ml. After 24 h, the medium was changed and 24 h later fibrates were added. The concentrations were 100 µM clofibric acid, 100 µM gemfibrozil, 10 µM fenofibrate, and 10 µM bezafibrate. Cells exposed to the solvent DMSO were used as negative control. The concentration of the solvent DMSO was 0.1%. RNA was extracted and reverse transcription was performed as mentioned above using 1000 ng total RNA.

Table 2. Primers used for expression analysis of PPARα, PPARβ, and PPARγ as well as the housekeeping gene β-actin. The optimal annealing temperature is 58°C.

Primer	Sequence	Size [bp]
PPARα_RT_FW	5'-GGAGTTCGCAAAGTCCGT-3'	166
PPARα_RT_RV	5'-GAACTCCCGGGTGATGAA-3'	
PPARβ_RT_FW	5'-CAATGCTCTGGAGTTGGATG-3'	161
PPARβ_RT_RV	5'- GAGTCAGAGTGGTTCGCTTG-3'	
PPARγ_RT_FW	5'-AGGCAGAGAAGGAGAAGCTG-3'	162
PPARγ_RT_RV	5'- GTCTTCCCAGAGAGGATTGC-3'	
β-actin_RT_FW	5'- TTGATCTTCATGGTGGATGG-3'	162
β-actin_RT_RV	5'-TCCACGAGACCACCTACAAC-3'	

For real time PCR, specific primers for all three receptors were designed, with annealing temperature at 58°C and a resulting product of approximately 160 bp (Tab. 2). β-actin was used as a housekeeping gene. Real time PCR amplification was performed on a RotorGeneTM 6000 (Corbett Life Sciences, Brisbane, Australia) using the FastStart SYBR Green System (Roche Diagnostics, Switzerland). The amplification conditions consisted of initial denaturation at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 45 sec, and elongation at 72°C for 45 sec. A melting curve analysis was performed after the run.

Fatty-Acyl CoA Oxidase (FAO) Assay

PLHC-1 cells were seeded on cell culture dishes (10 cm diameter) and 24 h later exposed to fibrates. The medium containing the fibrates was changed every day. After 72 h, the cells were harvested with a rubber policeman (VWR, Switzerland) and lysed with lysis buffer (60 mM Trisma Base/pH 8.3, 0.25 M Sucrose, 1% Triton X-100 containing one tablet of Protease Inhibitor Cocktail (Roche, Switzerland)) and by sonification twice for 15 sec (60%, 0.6 cycle). The lysate was centrifuged for 20 min at 15'000g at 4°C. The amount of protein was measured using the BioRad Protein Assay (Bio-Rad, Switzerland) based on the Bradford dye-binding procedure (Bradford 1976).

The FAO-assay (Small *et al.* 1985) was performed in a 96-well microplate. The reaction mixture contained 12 units/ml horseradish peroxidase, 0.05 mM di-chloro-di-hydro-fluorescamine and 40 mM aminotriazole in 10 mM PBS/pH 7.4, 0.6 mg/ml BSA and 0.02% Tween. Cell lysate was added to the reaction mixture with a final concentration of 400 µg/ml protein and preincubated for 5 min in the dark. The microplate was

measured at 502 nm on a spectrophotometer (Tecan, Switzerland). To start the reaction 30 µM palmitoyl CoA was added to each well and the enzymatic reaction was monitored by measuring at 502 nm every 5 min during 1 h.

Data analysis

Sequence manipulations, analysis and multiple alignments were done using BLAST Internet service, BioEdit (version 5.0.1) and DNASIS MAX (version 2.05, Hitachi Software Engineering Co.). Sequences were submitted to GenBank (NIH genetic sequence database) and got the accession numbers EU180568 (PPARα), EU180569 (PPARβ), EU180570 (PPARγ), EU180571 (RxR), and EU180572 (β-actin).

All experiments were independently repeated three times and the results of typical experiments are shown. Expression analysis was performed using Rotor-Gene 6000 Series Software (Corbett Life Sciences, Brisbane, Australia) and Excel. Data from the FAO-assay were analysed by one-way ANOVA (p-value < 0.05).

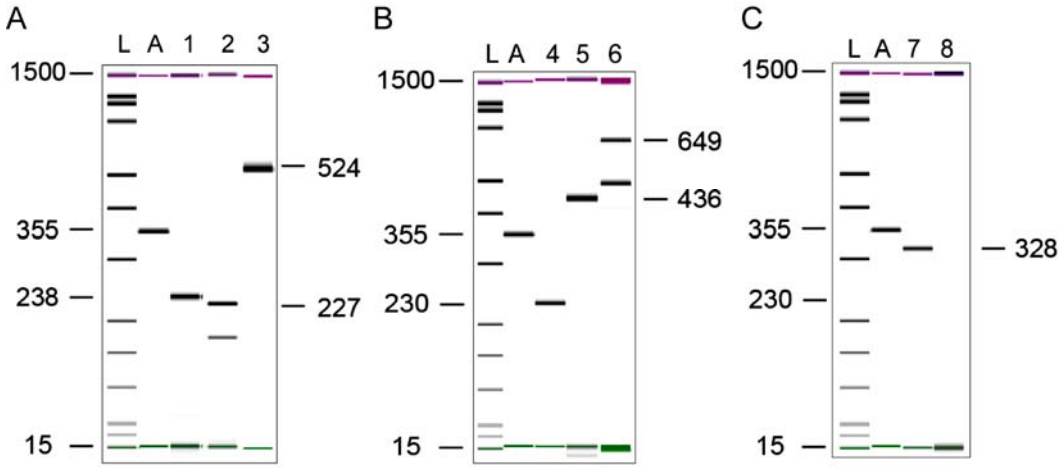
Results

Three PPARs and RxR are expressed in PLHC-1

Sequences for the four receptors PPARα, PPARβ, PPARγ and RxR are not kown for *P.lucida*, the organism PLHC-1 cells are derived from, but the receptors have been described in various other organisms including fish. Therefore, known sequences were aligned and degenerated primers were designed on conserved regions of each receptor. Several primers and combination of primers as well as different annealing temperatures were tested. Table 1 shows the primer pairs and the corresponding annealing temperatures that resulted in PCR products of the expected sizes (Fig. 1).

The three PCR products found for PPARα had sizes of 227 bp, 238 bp and 524 bp and the sequence showed a similarity of up to 86% (*Dicentrarchus labrax* and *Takifugu rubripes*) with other fish species (Fig. 1A). The sequence is a part of the ligand binding domain of the receptor an all helical domain. The translated sequence (Fig. 2A) revealed 82% identities and 92% positives with the human PPARα pointing at a high degree of conservation.

Figure 1: Results of RT-PCR for the three receptors PPARα (A), PPARβ (B), and PPARγ (C). PCR products were analyzed on a Bioanalyzer (Agilent Technologies, Switzerland). L: ladder; A: actin; 1: PPARα, fw/rv 1/1; 2: PPARα, fw/rv 2/2; 3: PPARα, fw/rv 2/3; 4: PPARβ, fw/rv 1/1; 5: PPARβ, fw/rv 2/2; 6: PPARβ, fw/rv 1/2; 7: PPARγ, fw/rv 1/1; 8: PPARγ, fw/rv 2/2



For PPARβ, PCR products were found of 229 bp, 448 bp and 636 bp with an overall similarity of up to 89% with *Dicentrarchus labrax*, *Pagrus major*, *Scophthalmus maximus* and *Oryzias latipes* (Fig 1B). A high conservation was also found for the 212 amino acid long translated sequence (Fig. 2B) that comprised the ligand binding domain. Identities were up to 96% and positives up to 98% with fish species (*Sparus aurata*, *Dicentrarchus labrax*, *Pagrus major*) and 82% and 89% identities and positives, respectively, with human PPARβ.

The smallest fragment was found for PPARγ with a total size of 313 bp equivalent to 104 amino acids (Fig. 1C). The cDNA identities were as high as 90% (*Dicentrarchus labrax*) and the translated sequence (Fig. 2C) showed 96% identities and 98% positives with *Platichthys flesus*. The sequence covers a part of one of the two zinc finger domains that are an element of the DNA binding domain. Conserved cysteins are found at positions 2, 12, 15 and 20.

Upon binding of a ligand, PPARs form a heterodimer with the retinoid X receptor (RxR). This complex binds to PPREs and activates the expression of target genes. Hence, RxR is a necessary prerequisite for the functionality of PPAR activation. A 530 bp partial cDNA was found with identities up to 88% to fish (*Paralichthys olivaceus*) and the translated sequence (Fig. 2D) that is a part of the ligand binding domain showed 87% identities and 93% positives with the human RxR receptors.

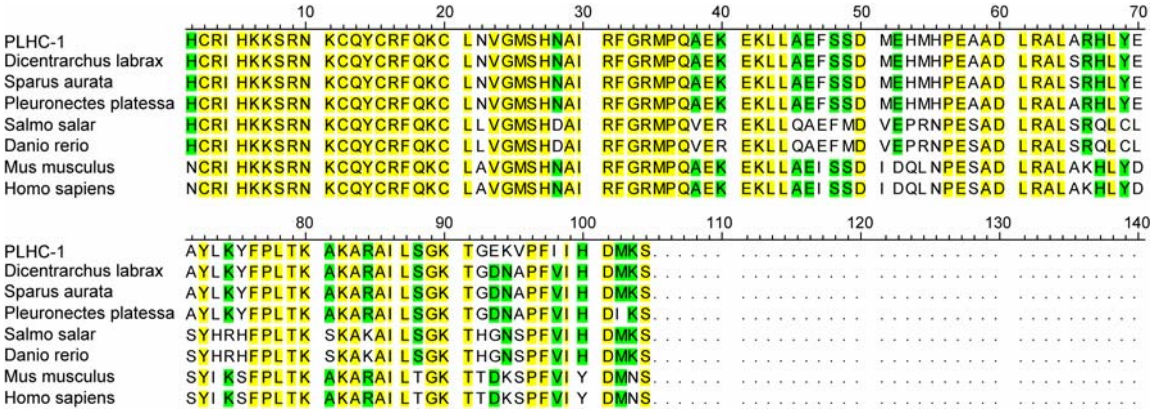
Alignments of all four receptors found in this study in PLHC-1 cells with fish sequences as well as human and mouse are shown in figure 2.

Figure 2: Sequence alignments for PPARα (A), PPARβ (B), PPARγ (C) and RxR (D). Sequnces were aligned using DNASIS MAX (Hitachi Software Engineering Co.). PPARα, PPARβ, and RxR comprise the all helical ligand binding domain. PPARγ covers the last part of one of the two zinc finger domains that are an element of the DNA binding domain. Conserved cysteins are at position 2, 12, 15, and 20. Yellow: 100% identity, green: > 50% identity.

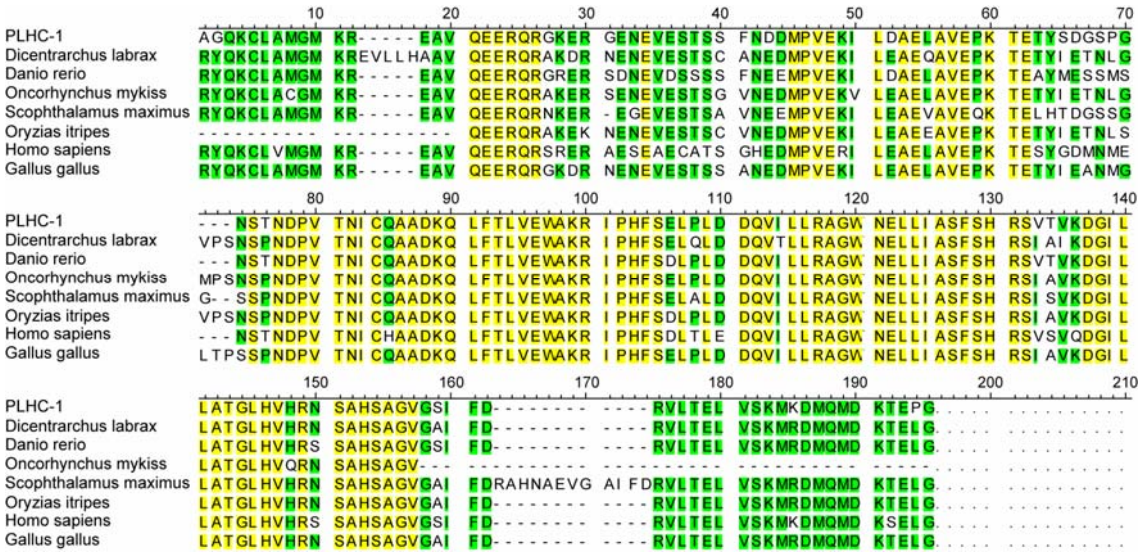
A)

B)

C)



D)

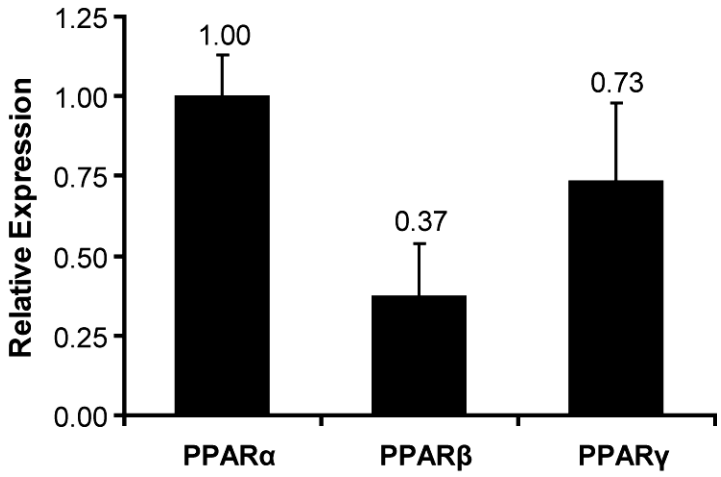


Expression analysis of PPARs

Specific primers for expression analysis were designed based on the obtained sequences (Tab. 2). All primer pairs have an optimal annealing temperature of 58.0°C and give a PCR product between 161 bp and 166 bp. First, the relative expression of the three receptors was assessed. The highest expression level was found for PPARα (100%) followed by PPARγ (73% of PPARα). PPARβ was expressed 3 times less than PPARα (37%) (Fig. 3).

To assess an induction of the PPAR-pathway, the three fibrates bezafibrate, fenofibrate and gemfibrozil as well as clofibric acid, the active metabolite of several fibrates, were chosen as they are ligands and inducers of mammalian PPARs. Confluent PLHC-1

Figure 3: Expression analysis of the three PPARs in PLHC-1 cells. The expression is indicated relative to PPARα. Shown are means ± standard deviation (n=3).



cells were exposed to the different fibrates or the solvent control. An induction of PPARα and PPARγ was found for all four fibrates after 6 h of exposure, the expression level of PPARβ was not changed, however (Fig. 4B). Bezafibrate and clofibric acid revealed the strongest induction with 4.03-fold and 2.77-fold for PPARα and 10.45-fold

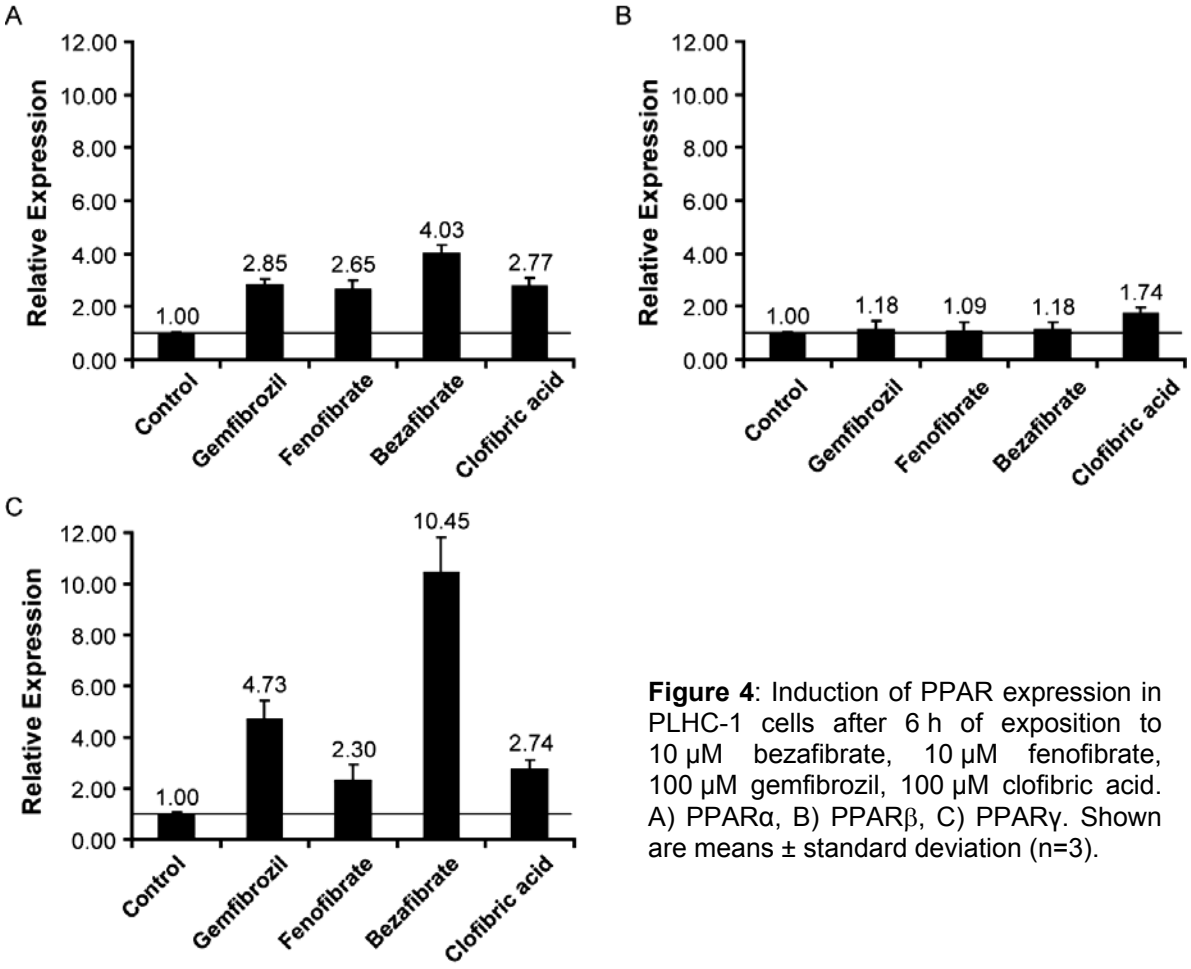


Figure 4: Induction of PPAR expression in PLHC-1 cells after 6 h of exposition to 10 µM bezafibrate, 10 µM fenofibrate, 100 µM gemfibrozil, 100 µM clofibric acid. A) PPARα, B) PPARβ, C) PPARγ. Shown are means ± standard deviation (n=3).

and 2.74-fold for PPARγ (Fig. 4A/C), respectively. The exposure to fenofibrate resulted in a 2.65-fold and 2.30-fold induction of PPARα and PPARγ, respectively. Gemfibrozil was slightly more potent than fenofibrate. The induction of PPARα was 2.77-fold and of PPARγ 2.81-fold. However, no significant induction was found in cells that were exposed for 3 d, 7 d and 14 d to fibrates (data not shown).

FAO activity

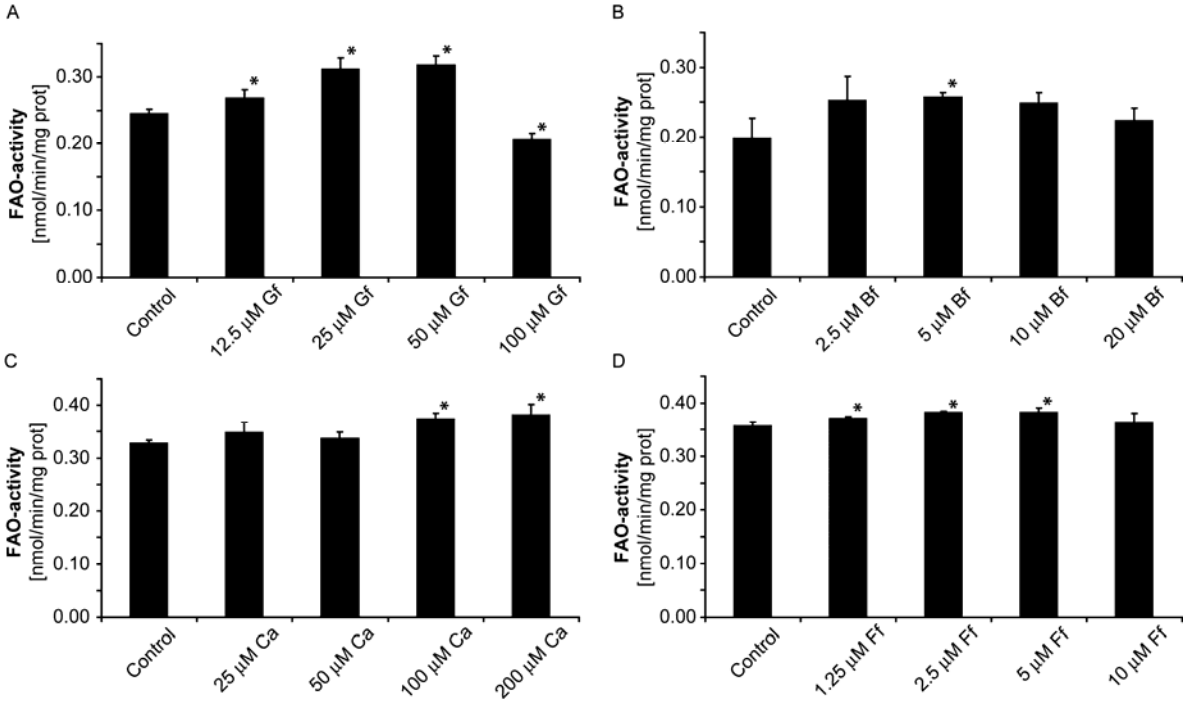
As a consequence of peroxisome proliferation peroxisomal enzymes and proteins become higher expressed. FAO is an enzyme involved in the β-oxidation of fatty acids in the peroxisome. We exposed PLHC-1 cells to different concentrations of bezafibrate, clofibric acid, fenofibrate, and gemfibrozil and measured the FAO activity after 72 h. All fibrates showed an increased dose-dependent activity (Fig. 5). The most potent fibrates were gemfibrozil and bezafibrate with a maximal increase in FAO activity of 30% at a concentration of 50 µM and 5 µM, respectively. Clofibric acid showed a 16% higher activity than controls and the least effects were found for fenofibrate with a 7% increase at 2.5 µM.

Discussion

Our work describes for the first time the presence and functional activity of PPARs in an *in vitro* model system for aquatic organisms, the PLHC-1 cell line. A response by the PPAR pathway is only possible when the activated PPAR forms a heterodimer with the RxR receptor (Escher and Wahli 2000). We detected all three PPARs as well as RxR demonstrating that the prerequisite for a response by the PPAR pathway is given in the PLHC-1 cells. In addition, after exposure of fibrates to PLHC-1 cells, an induction of the expression of PPARα and PPARγ was found as well as an increased activity of the peroxisomal enzyme FAO that is involved in the β-oxidation pathway of fatty acids.

We detected four partial sequences: The sequence of PPARγ covered the last part of one of the two zinc finger domains that are an element of the DNA binding domain. As expected, the sequence is highly conserved. The sequences of PPARα, PPARβ and RxR covered the all-helical ligand binding domain. The high similarities with the corresponding receptors of other fish species and even with humans indicate that these receptors still have the same or at least similar ligand specificities and, consequently,

Figure 5: Increase in FAO activity after 72 h exposition to fibrates. A) Exposition to gemfibrozil (Gf); B) exposition to bezafibrate (Bf); C) exposition to clofibric acid (Ca); D) exposition to fenofibrate (Ff). Shown are means ± standard deviation (n=3). Significant values are marked with * ($P < 0.05$).



similar roles within the cell. However, there are studies that show differences between mammalian and fish PPARs. Potent activators of mammalian PPARβ and PPARγ failed to induce these receptors of *Takifugu rubripes* in a reporter assay (Kondo *et al.* 2007). Other studies found that synthetic compounds and fatty acids induced the transcriptional activities in *Pleuronectes platessa* and *Sparus aurata* of PPARα and PPARβ, identical as in other vertebrates. However, the activity of their PPARγs was not enhanced by these products (Leaver *et al.* 2005). Another difference found in fish is the numbers of PPARs. One of each of the three PPARs has been reported in mammals. However, there have been found four (two isoforms of PPARα) in *Takifugu rubripes* (Maglich *et al.* 2003). In addition to one PPARγ, *Danio rerio* has two PPARα as well as two PPARβ (Robinson-Rechavi *et al.* 2001). Even four PPARβ subtypes were recently described in Atlantic salmon (*Salmo salar*) assigned into two families, each containing two isotypes (Leaver *et al.* 2007).

The expression level of PPARs has been investigated after exposure to ligands such as fibrates in *Salmo salar* and in *Carrassius aurata* (Mimeault *et al.* 2006; Ruyter *et al.* 1997). A 1.8-fold increase in PPARγ expression was found after 3 d exposure to 0.5 mM clofibric acid as well as 0.5 mM bezafibrate in *Salmo salar* (Ruyter *et al.* 1997).

Mimeault et al. (2006) found a decrease of the PPARβ expression level after 14 and 28 days of exposure to 1500 µg/L (6 mM). The expression levels of PPARα and PPARγ were unchanged, however. We have found an increased expression of PPARα and PPARγ in PLHC-1 cells after exposure for 6 h to all four fibrates of up to 10-fold. However, no significant induction was found in cells that were exposed for 3 d, 7 d and 14 d to fibrates (data not shown). The expression level of PPARβ remained unchanged. We assume that the fibrates bezafibrate, fenofibrate, gemfibrozil and the metabolite clofibric acid activated PPARα and that PPARα itself as well as PPARγ are regulated by PPARα. There is evidence that the expression of PPARα and of PPARγ is induced in the beginning upon activation of PPARα as first response and turns back to basal level later on. Our results suggest that PPARβ is not regulated by PPARα in the PLHC-1 cells. In summary we found in PLHC-1 cells that all fibrates designed to be ligands for PPARα enhanced the expression of PPARα and PPARγ. Further investigations with PPARβ and PPARγ specific ligands are necessary to characterize the functionality of these two receptors in the PLHC-1 cells.

The FAO activity has widely been used as a measure of peroxisome proliferation (Cajaraville *et al.* 2003; Donohue *et al.* 1993; Ibabe *et al.* 2005a; Mimeault *et al.* 2006; Ortiz-Zarragoitia and Cajaraville 2005; Ruyter *et al.* 1997). In our study, the FAO activity turned out to be not very sensitive to the exposure of fibrates. We have found a maximal increase in FAO activity of up to 30% over controls. The next step is to investigate effects on the expression level of different peroxisomal enzymes like FAO, bifunctional enzyme, and thiolase, which is probably a more sensitive approach.

There are an increasing number of compounds reported to occur in the aquatic environment. Some of them such as pharmaceuticals, are known to have a specific mode of action in mammals and are assumed to act similarly in aquatic organisms (Fent *et al.* 2006; Owen *et al.* 2007). Other substances have so far not been characterized and may have the potential to exhibit unexpected effects. In surface waters, there is usually a complex mixture of different compounds present and therefore the risk of mixture effects arises. To address this issue it is necessary to first understand the biochemical mechanisms in aquatic organisms and to develop appropriate tools to screen for effects of numerous single substances, but also of complex (environmental) mixtures. For this purpose, *in vitro* systems are promising

tools as they are easy to handle and produce more reproducible results than *in vivo* systems.

For our study we have chosen the fish hepatoma cell line PLHC-1 as it has widely been used in ecotoxicology as an *in vitro* model system to study detoxification mechanisms involving phase I and phase II enzymes (Fent 2001). The induction of the phase I enzyme CYP1A1 has been used for the assessment of the toxic potential of polycyclic aromatic hydrocarbons (PAHs), for single substances but also for complex environmental mixtures (Fent and Batscher 2000). Recently, the two efflux transporters P-gp1 (ABCB1) and MRP3 (ABCC3) that export a wide range of xenobiotics out of the cells have been detected and functionally characterized (Caminada *et al.* submitted; Zaja *et al.* 2007). Furthermore, the PLHC-1 cells have been used for the study of heat shock proteins (Ryan and Hightower 1996), metallothionein induction (Schlenk and Rice 1998) and cytotoxicity (Caminada *et al.* 2006). Due to the presence of all three critical phases of the detoxification system the PLHC-1 cells have been proposed as a reliable *in vitro* model in aquatic toxicology (Zaja *et al.* 2007). So far, only the aryl hydrocarbon (Ah) receptor has known to be present and functional in the PLHC-1 cells. Our present study demonstrates that there are at least four more nuclear receptors present in PLHC-1 cells, which are functional. These findings make the PLHC-1 cells an even more interesting model system for the investigation of molecular mechanisms of environmental pollutants in aquatic systems.

Our findings correlate also with those found in fathead minnow (*Pimephales promelas*) where we investigated effects of bezafibrate and clofibric acid after chronic exposure for 14 days and 21 days. The expression level of PPARα was not affected significantly after 14 and 21 days of exposition. In contrast, the FAO activity after exposition for 14 days to clofibric acid was about 1.5 times higher in male fish than in controls (Weston *et al.* submitted).

In conclusion, our study shows for the first time the presence of all three PPARs as well as the RxR in PLHC-1 cells. We confirm previous suggestions that this cell line is an appropriate *in vitro* model system for aquatic organisms. Furthermore, the fibrates bezafibrate, fenofibrate, gemfibrozil and clofibric acid induced the expression of PPARα as well as PPARγ and they increased also the activity of FAO. In surface waters, the maximal concentrations of these fibrates are 4 µg/L. The effects found in this *in vitro*

study were at concentrations between 1.8 mg/L to 25 mg/L, about a factor 1000 higher than environmental concentrations. Whether *in vitro* effects in fish cells are less sensitive than effects *in vivo* has to be further analyzed. Further studies are also necessary to assess the specificity of PPARs towards other environmental pollutants and to estimate effects of compound mixtures.

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CHAPTER 6

Effects of bezafibrate and clofibric acid on fathead minnows; study on peroxisome proliferator activated receptor alpha and related proteins in liver

Anna Weston[a,b], Daniel Caminada[a,c], Hector Galicia[b] ,Karl Fent[a,d]

[a] University of Applied Sciences Northwestern Switzerland, School of Life Sciences, Institute of Ecopreneurship, CH-4132 Muttenz, Switzerland

[b] Springborn Smithers Laboratories (Europe), CH-9626 Horn, Switzerland

[c] University of Zurich, Institute of Plant Biology, Division of Limnology, CH-8802 Kilchberg, Switzerland

[d] Swiss Federal Institute of Technology (ETH), Department of Environmental Sciences, CH-8092 Zurich, Switzerland

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Abstract

Human pharmaceuticals are a class of emerging environmental pollutants, which are designed to act on specific targets. Our interest focuses on the lipid lowering agent bezafibrate and clofibric acid that, among other natural ligand and xenobiotics, are involved in peroxisome proliferation. We analysed the effects on liver metabolisms of fish after 14 and 21 days exposure to several concentrations from 0.1 to 106.7 mg/L bezafibrate, and 1.07, 10.75 and 108.91 mg/L clofibric acid, respectively, focussing in particular on peroxisome proliferator activating receptors and related enzymes. In addition we determined reproductive parameter such as egg production and vitellogenin induction to test the hypothesis, whether these human pharmaceuticals may be also linked to reproductive and developmental toxicity. We investigated whether both the expression of PPARα and the fatty-acyl oxidase (FAO) activity are affected in liver of fathead minnows. No effects were found in fish exposed to bezafibrate, in both mRNA expression of PPARα and FAO level at all concentration tested. Male fathead minnows exposed to clofibric acid showed an increased in FAO activity at 108.91 mg/L. This was not found in females and could not be related to an increase in PPARα expression. In addition a non significant decrease in egg production after 21 days of exposure to 1.07- 108.91 mg/L clofibric acid was found. In this study we demonstrate that the two lipid lowering agents did not significantly affect the parameter investigated at the concentrations tested, nor at environmentally relevant concentrations.

Key words: Peroxisome proliferation, PPAR, fish, fibrates

Introduction

The awareness of potential environmental risks of human pharmaceuticals has increased in the last decade, as a considerable number of diverse medicinal products have been detected in the aquatic environment with concentrations that may reach several µg/L. Pharmaceuticals are designed to elicit a biological response in humans, but for most of these contaminants, potential toxic effects on aquatic organisms are not known, in particular after long-term exposure. In recent years it has become increasingly clear that it is not sufficient for the assessment of ecotoxicological hazard and risks to assess acute toxicity only. It is important to investigate the possible chronic effects of human pharmaceuticals on aquatic organisms. In addition, it is important to investigate these effects by focussing on target biomolecules and the histopathology of the organs (Fent *et al.* 2006). This was demonstrated for the non-steroidal antiinflammatory compound diclofenac, affecting liver and kidneys in fish similarly as in humans (Schwaiger *et al.* 2004). We proposed a concept of toxicity evaluation of pharmaceuticals in aquatic organism by focussing on their mode of action (Fent *et al.* 2006). In regarding the mechanisms of action that may be similar in mammals and fish, more information on adverse effects may be gained than performing classical toxicity tests. Therefore, analysis of ecotoxicological effects should be performed following a mechanism-based concept.

Amongst the most ubiquitously pharmaceuticals found in aquatic environment are the blood-lipid lowering agents bezafibrate and clofibric acid, the latter being the main metabolite of clofibrate, etollynifibrate and etofibrate. Bezafibrate occurred in sewage treatment plants effluents and surface water with maximal concentrations of 4.6 µg/L (Ternes 1998) and 3.1 µg/L (Stumpf *et al.* 1996). Clofibric acid has been found in sewage treatment effluents at maximal concentration of 1.6 µg/L (Ternes 1998) and at a maximal concentration of 0.55 µg/L in surface water (Buser *et al.* 1998). So far, for bezafibrate only acute toxicity data in *Daphnia magna* (EC_{50}>200 mg/L) has been reported (Hernando *et al.* 2004). Clofibric acid has been analysed only for subchronic toxicity with LOEC for algae of 150 mg/L, for *Cerodaphnia dubia* of 2.56 mg/L and of 140 mg/L in early life stage test of zebrafish (Ferrari *et al.* 2003). Acute toxicity on different organisms (Nunes *et al.* 2004) including oxidative stress has been reported (Nunes *et al.* 2006). In rats, fibrates have been found to induce peroxisome proliferation, which is strongly correlated to hepatocarcinoma (Qu *et al.* 2001). However

this has not been demonstrated in primates or fishes. As in mammals fibrates were found to interact with peroxisome proliferator-activated receptors (PPARs) in fish (Cajaraville *et al.* 2003; Ibabe *et al.* 2005; Ruyter *et al.* 1997) and mussels (Cajaraville *et al.* 2003). PPARs belong to the nuclear receptor superfamily and dimerize with the retinoid-X receptor to promote the expression of several lipid regulatory proteins by binding to a regulatory region of the target gene (Staels *et al.* 1998). PPARs are known to stimulate the proliferation of peroxisomes (Cajaraville *et al.* 2003; Escher and Wahli 2000), and as a consequence the activity of some of the specific peroxisome proteins is increased. Three subtypes of PPARs have been described in diverse animals such as mammals (Schoonjans *et al.* 1996), amphibians (Dreyer *et al.* 1992) and fishes (Andersen *et al.* 2000; Leaver *et al.* 1998; Ruyter *et al.* 1997). PPARα, PPARβ or δ and PPARγ, which different functions have been associated to, different tissue distributions in fishes has been described. In fish PPARα was mainly expressed in tissues catabolizing fatty acids such as liver, proximal tubules of kidney and enterocytes, whereas PPARβ is less specifically expressed, being found in different tissues (Ibabe *et al.* 2002). An increase in the PPARγ mRNA expression was demonstrated in hepatocytes of salmon exposed to fibrates (Ruyter *et al.* 1997). Besides, some organochlorine pesticides (Cajaraville *et al.* 2003), plasticizers (Ortiz-Zarragoitia and Cajaraville 2005) and perfluorooctanoic acid (Oakes *et al.* 2004) may activate PPARs in fish both *in vivo* and *in vitro*, therefore increasing the interest of using PPARs as biomarker for environmental pollution.

The fatty acyl-CoA oxidase (FAO) is an enzyme found only in peroxisomes and it plays a key role in the β-oxidation of fatty acid. An increased activity of the FAO has been found in primary hepatocytes of salmon (*Salmo salar*, (Ruyter *et al.* 1997)) and in rainbow trout and Japanese medaka (Scarano *et al.* 1994) after exposure to fibrates. The specific peroxisome membrane protein PMP70 was also found to be increased after exposure to a peroxisome proliferator in fish (Ackers *et al.* 2000). In a different study performed in our laboratory on fish hepatocytes we found a light increase in FAO activity after 72 hours exposure to 5 µM of bezafibrate and 100 µM of clofibric acid, respectively as well as a first increase in PPARα expression after 6 hours exposure to bezafibrate and clofibric acid, followed by a recovery of this receptor after 7 to 14 days (Caminada *et al.* submitted).

In our present study we investigate effects of bezafibrate (BF) and clofibric acid (CA) on fathead minnows (*Pimephales promelas*) after chronic exposure for 14-21 days by focussing on known target molecules of fibrates in order to evaluate the potential ecotoxicological effects of this important group of pharmaceuticals. Effects on PPARα expression and on FAO activity were determined after 14 or 21 days of exposure to different concentrations of these compounds in three different experiments. The aim was to better understand potential ecotoxicological effects of the blood-lipid lowering agents bezafibrate and clofibric acid on fish, and in addition, the role of their target biomolecules as potential biomarkers for environmental pollution.

Materials and methods

Chemicals

Bezafibrate (purity > 99%) was kindly supplied by F.Hoffmann-La Roche Ltd. (Basel, Switzerland). Clofibric acid (>99 %), fenoxycarb (>98 %) and dibutyl phthalate (DBP>98 %) were purchased from Sigma-Aldrich (Buchs, Switzerland). Ethanol and DMSO were purchased from TJ Backer (Backer Ltd, Switzerland). Chemicals used in the different assays are described below in the specific chapters.

Analytical determinations

Analysis of experimental water samples was performed for all experiments. For fish in experiment A different volumes of samples were extracted using a Strata Screen-A cartridge (500 mg/6 mL, 8B-S019-HCH, Phenomenex). Conditioning and equilibration were performed prior to sample loading with 5 mL of MeOH and 5 mL 50 mM phosphate buffer (pH 7), respectively. The rate of sample loading was kept at 4-8 mL/min. The analytes were eluted with a solution of 1 % concentrated HCl in 70 % 1:1 MeOH/ACN. The extracts were dried under a stream of N_2 at 40°C for 1-1.5 h, and at 55°C for 1-2 h, and subsequently dissolved with 1 to 2 mL of 40 % ACN/MilliQ water. For experiment B and C the samples were either diluted 1 : 10 (CA or BF 100 mg/L) or directly analysed (CA 1 and 10 mg/L) without prior extraction.

20 µL of extracts or sample were injected in a HPLC (Hewlett Packard HP 1090 or 1050), both equipped with a Spherisorb RP-C18 250 x 4.6 mm column, with a flow rate of 1 mL/min. The mobile phase consisted of a binary mixture of MilliQ water and

acetonitril. The linear gradient program began at 5 % ACN hold for 1 minute to end at 40 % ACN after 8 minutes. The peaks were detected after 8 minutes for CA and after 11.5 minutes for BF and were determined by UV detection at 225 nm.

Fathead minnows

Acclimatisation: Adult fathead minnows (*Pimephales promelas*) for experiment A were purchased from Osage Catfisheries Inc. (Osage Beach, MO, USA) and were kept in a water tank (300 L) for 3 weeks for acclimatisation prior to the beginning of the experiment. Two batches of fishes were delivered and kept separately. Fish of one batch were raised in the laboratory (Lab reared fish, LR), whereas the other batch was raised in an outdoor pond (pond fish, P) until delivering. Both batches were kept in the laboratory for acclimatisation prior to the experiment, each batch in a separate 300 L water tank. Fathead minnows for experiments B and C were cultured in house (Springborn Smithers). Temperature (25±2°C) and daylight photoperiod (16 h) were held constant during the acclimatisation period. The water tanks were supplied with a carbon filtration system.

Experimental design

The experimental procedure was the same for all the experiments. It was adapted from Kunz et al. (2006) and conducted by a semi-static procedure (Kunz and Fent 2006). For the experiments, reconstituted tab water with alkalinity range of 25-30 mg/L $CaCO_3$ and conductivity of 500 µS/cm was used. The experiments were conducted at a constant temperature of 25 ± 1°C. BF solutions were prepared the day prior to the water renewal and kept under agitation overnight, whereas CA solutions were freshly prepared the day when water renewal took place. Every 48 h, aquaria containing BF, CA or DHP were completely renewed. After 24 h a known volume of solution was soaked and renewed with fresh solution to eliminate food residuals and faeces. In each aquarium two steel tunnels were added. Fishes were fed twice daily with commercial flakes (Tetramin, Germany). Mortality, swimming behaviour as well as eggs production were recorded daily. Dissolved oxygen and pH were recorded twice during the renewal from fresh (0 h) and old solution (48 h). A number of 12 male, 6 male and 6 female for experiments A and C, and 8 fishes, 4 male and 4 female, in experiment B were randomly distributed in stainless steel aquaria containing BF, DBP, CA solutions or control water. The aquaria were filled with 15 L water containing different concentration of BF and CA or water control for experiment A and C, and with 10 L for experiment B.

Plastic top covers were used to avoid aeration, and air was delivered through air pumps connected to an appropriate plastic tube ending in a Pasteur pipette that was dept into water.

Experiment A: For each batch of fish (P and LR) two replicates of four concentrations of bezafibrate with a separation factor of 10 were applied. As a positive control, dibutyl phthalate (DBP 0.5 mg/L prepared in < 0.01 % DMSO) was chosen according to effects on FAO activity found in the literature (Ortiz-Zarrgoitia et al. 2005). The experiment was conducted in a climate chamber and exposure lasted 14 days.

For analysis of actual BF concentrations, 1 L of each water samples were collected of control and the different BF dose groups at time 0 and 48 hours during the 14 days exposure period. This occurred at day 0, 2, 6, 8, 10, 12, whereas aliquots of the fresh stock solution of 100 mg/L were collected at each water renewal. Water samples were extracted and analyzed as described above.

Experiment B: Two replicates of 100 mg/L bezafibrate and two replicates of a water control were applied. During the 14 days of exposure the aquaria were kept in a water bad at 25°C.

For chemical analysis of actual BF concentrations, 100 mL of each water samples were collected of control and the 100 mg/L BF dose group at time 0, 24 and 48 hours. This occurred at day 0 and 8 during the 14 days exposure period. Control water samples were directly injected to the HPLC for analysis, whereas bezafibrate samples were diluted 1 : 10 with fish medium prior to injection.

Experiment C: Three replicates of 1, 10 and 100 mg/L of CA and three replicates of a water control were applied. The experiment was conducted in a climate chamber were temperature was kept at 25°C. The exposure lasted for 21 days.

For analysis of actual CA concentrations, 50 mL of water were collected of control and the different CA dose groups at time 0, 24 and 48 hours during the 21 days exposure period. This occurred at day 0, 2, 6, 8, 10, 12. Controls and samples from doses CA 1, and 10 mg/L were directly injected to the HPLC for analysis, whereas from the dose CA 100 mg/L samples were diluted 1 : 10 with tap water prior to injection.

Termination of experiments: At the end of the exposure period, the fishes were anesthetized with 100 mg/L MS-222 (tricaine, Fluka) and the lengths and the weights were measured. Blood was collected with 20 or 50 µL heparinised capillary tubes (KABE Labortechnik GmbH, Nümbrecht-Elsenroth, Germany) from the caudal vein and kept on ice for a maximum of 3 hours. 20 µL at 2 units/mL of protease inhibitor (Aprotinin, Fluka AG, Buchs, Switzerland) were added to each blood sample. Plasma was collected after centrifugation (10 min at 3000 rcf and 4°C) and stored at -80°C until analysis. Fishes were then dissected to collect livers. Depending on the analysis to be performed, livers were either directly fast-frozen in liquid nitrogen for FAO activity measurement or given to a RNA later solution (Qiagen, for quantitative rtRT-PCR) which was let soak into livers overnight at room temperature according to the manufacture protocol. All livers were then stored at -80°C until analysis was performed.

Vitellogenin, FAO activity and PPARα expression:

Vitellogenin determination: Vitellogenin analysis was performed with a commercial ELISA kit (Biosense Laboratories, Bergen, Norway) in experiment A following the instruction of the manufacturer. The kits were provided with monoclonal antibodies against vitellogenin in order to perform a sandwich ELISA analysis. Briefly, purified vitellogenin supplied in the kits was freshly prepared every day of analysis as standard for vitellogenin quantification. Fish plasma samples were first diluted with sample buffer delivered with the commercial kit to achieve a 1:2 ratio of plasma/buffer. Further dilution steps were prepared as follows: 1:5000; 1:500000; 1:5000000 for female plasma and 1:50; 1: 5000; 1:500000 for male plasma. Measurements were performed in duplicate and colour was measured at 450 nm in a spectrophotometric microtiter plate reader (Tecan, Infinite M200, Switzerland). As first screening fish from water control, positive control and 100 mg/L BF dose were analysed.

FAO activity. The activity of fatty acyl oxidase (FAO) was measured according to the method of Small (Small *et al.* 1985), with some minor modifications of the protocol (Ortiz-Zarragoitia and Cajaraville 2005; Ruyter *et al.* 1997). Livers were let thaw on ice, weighed with an analytical scale and placed in 100 µL of ice cold buffer (0.6 M TRIZMA base, 0.25 M sucrose, 0.01 % TritonX-100) and a protease inhibitor cocktail (F.Hoffmann-La Roche, Switzerland). Livers were homogenised with an ultrasonic homogenisator (Labsonic, Satorius AG, Germany) and supernatants were collected

after centrifugation at 600 rcf for 20 minutes. Before starting the reaction, the amount of proteins was measured by a BioRad protein assay. Briefly, liver samples were prepared at 400 µg/mL protein concentration in a dilution buffer (PBS at pH 7.4 with 0.02 % Tween and 0.6 mg/mL BSA) and added in triplicate on a microtiter plate. 40 µL of reagent mixture (12 units/mL horseradish peroxidise, 0.05 mM 2',7'-dichlorodifluorescein diacetate, 0.015 mM FAD and 40 mM aminotriazole, all chemicals purchased from Sigma-Aldrich, Buchs, Switzerland) were then added to each sample, and incubated in the dark for 5 minutes prior to measurement at 502 nm. 10 µL of palmitoyl CoA (Sigma-Aldrich, Buchs, Switzerland) at a final concentration of 30 µM were then added to start the reaction and the kinetics of the enzyme (product formation) was monitored for 1 hour (13 cycles of 5 minutes) at 502 nm using a spectrophotometric microtiter plate reader (Tecan, Infinite M200, Switzerland). A serial dilution of dichlorofluorescein standard was used to quantify the amount of dichlorofluorescein produced from dichlofluorescein diacetate during the reaction. The FAO activity is expressed as nM DCF/min*mg protein.

PPARα expression: RNA were extracted from liver samples with a commercial kit (RNeasy extraction kit, Qiagen Switzerland) and according to the manufacture protocol. Extracted RNA were resuspended in 35 to 45 µL of nuclease free water (Qiagen, Switzerland). The reverse transcription reaction was carried out according to the manufacturer protocol with a reverse transcriptase (Roche Diagnostic, Switzerland) The same initial concentration of 0.1 µg/µL RNA was taken for each sample to be transcribed, and was ensured by a spectrophotometric measure of RNA concentration at 260 nm (NanoDrop ND-1000 full-spectrum (220-750 nm) spectrophotometer, Witec, Switzerland). For the reverse transcription, polydT primer (Roche Diagnostic, Switzerland) were used for a non-specific transcription of whole RNA into cDNA. A first set of degenerated primers previously designed for PLHC-1 cell was used to clone the PPARα and the β-actin cDNA of fathead minnows (Caminada et al. submitted), which were subsequently sequenced (Synergene Biotech GmbH, Switzerland) and submitted to gene bank (accession number EU195886 and EU195887, respectively). The purification procedure for sequence analysis is described elsewhere (Caminada et al, submitted). According to the sequence obtained, a second set of primer was designed to get a shorter band for each gene for the quantitative PCR. The sequences of the primers were 5'-GCGTCCTGCATGAATAAAGA-3', 5'-GTCCAGCTCGAGAGCGTT-3'for PPARα, and 5'-TCCGTAAGGATCTGTATGCC-3', 5'-

Table 1: Summary of experiments performed and endpoints analyzed.

	Exp. A	Exp. B	Exp. C
Number of fish	12	8	12
Exposure (days)	14	14	21
Volume of sample (L)	15	10	15
Substance	BF	BF	CA
Replicate	4	2	3
Nominal concentrations (mg/L)	0.1/1/10/100	100	1/10/100
FAO	√	-	√
PPARα	-	√	√
Vitellogenin	√	-	-

GATCCAGACGGAGTATTTGC-3' for β-actin (Microsynth, Switzerland). These set of primers were tested with standard Corbett PCR machine (45 cycles of 30 seconds at 94°C, 45 seconds at 55°C and 45 seconds at 72 °C) and analyzed on a DNA chip 1000 (Bioanalyzer, Agilent technologies, Switzerland) prior to start with the quantitative PCR analysis. The bands were detected at 158 bp for β-actin and at 150 bp for PPARα. No unspecific amplifications seemed to occur.

After reverse transcription the resulting cDNA samples were then diluted for real time analysis. A standard dilution sequence (1 : 20 to 1 : 5120, with a factor 4 of separation between each step) as well as a 1 : 320 dilution of each sample were prepared for both β-actin (housekeeping gene for normalization) and PPARα. The fluorescent dye FastStart SYBR Green (Roche Diagnostic, Switzerland) was chosen for the real time procedure. The real time PCR program was performed with 50 cycles and an annealing temperature of 58 °C for 45 seconds. At the end a melting procedure for quality analysis was also performed between 50 and 95 °C, and 1°C/5 seconds. All the real time reactions were performed on a Corbett RotorGene TM 6000 (Corbett Life Sciences, Australia).

Table 1 gives a summary of the experiments performed and the respective endpoints analyzed.

Statistical analysis:

An analysis of variance test (ANOVA) was performed to determine the effects of bezafibrate and clofibric acid on investigated parameter. An effect was chosen as significant if $p<0.05$.

Results

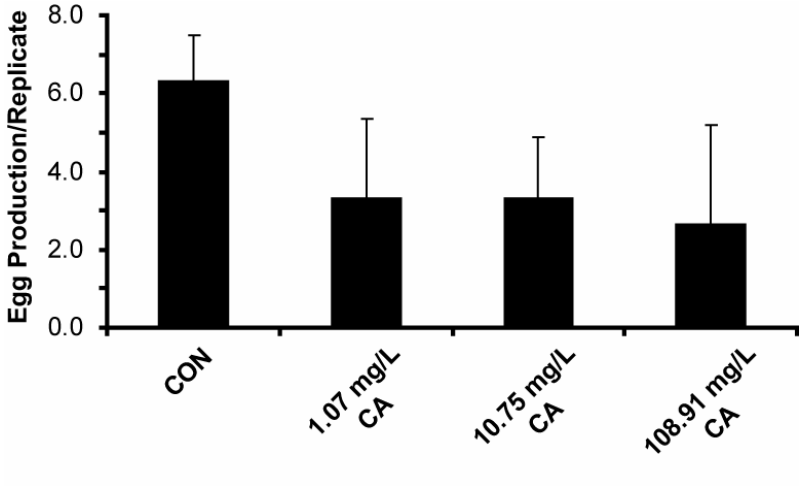
Chemical analysis: The chemical analysis of water sample of all fish experiments showed that the concentrations of BF and CA were close to nominal values and did not decrease during the renewal period of 48 hours. This was the case in all the treatments ($p>0.05$). Therefore mean levels in each dose group were taken as exposure concentrations. The analysis of water control did not show any contamination.

Weight, length and egg production: Both the fibrates analyzed in this study did not interfere nor with the weight nor the length of fathead minnows. In Table 2 the effects on condition factor ($CF=10^5*W/L^3$, where W is the weight of the fish expressed in grams, and L is the length expressed in millimetres) are summarized for fish exposed to BF and CA, respectively. As both adult female and male fish were kept in aquaria, mating could take place Fish exposed to 101.56 mg/L BF in experiment A produced eggs just twice (data not shown). Also in experiment B fish of all groups did not produced enough eggs to perform a statistical analysis. Here the sporadic egg production was found in different groups, but the egg production events were lower than 5 times (data not shown). Interestingly, by fish exposed to CA the egg laying seemed to be affected, even if this effect was not significant (Figure 1). Fish exposed to 1.07 and to 10.75 mg/L CA showed a tendency to decreased egg production ($p=0.052$), whereas fishes exposed to 108.91 mg/L CA did not significantly differ compared to control fish ($p=0.08$). This lack of significance may be explained by the

Table 2: Condition factors of experiments A, B and C represented as mean±SD of male and female fish. Difference between male and female at 10.75 mg/L CA is significantly increased for male (**p=0.03). BF – Bezafibrate; CA – Clofibric acid; DBF-dibuthyl phthalate 0.5 mg/L.

Exp.		Male	Female
A	CON	1.073 ± 0.096	1.142 ± 0.226
	DBP 0.5	1.035 ± 0.040	1.114 ± 0.155
	BF 0.1	0.970 ± 0.027	1.077 ± 0.127
	BF 1.27	1.017 ± 0.183	1.148 ± 0.138
	BF 10.18	0.944 ± 0.043	1.054 ± 0.115
	BF 101.56	0.953 ± 0.086	1.030 ± 0.150
B	CON	0.984 ± 0.004	1.021 ± 0.024
	BF 106.7	1.016 ± 0.080	0.995 ± 0.005
C	CON	1.164 ± 0.062	1.172 ± 0.084
	CA 1.07	1.128 ± 0.025	1.104 ± 0.007
	CA 10.75	1.232 ± 0.029	1.092 ± 0.072**
	CA 108.91	1.150 ± 0.027	1.091 ± 0.050

Figure 1: Egg production of control fish (CON) and fish exposed to three different concentrations (1.07 mg/L, 10.75 mg/L, 108.91 mg/L) of clofibric acid (CA) during 21 days. Statistically not significant (p>0.05 for all the treatments).



fact that, out of three replicates just two produced eggs during the exposure period of 21 days, whereas in the control fish egg production took place in all three replicates. Table 3 summarizes data on egg production in experiment C including the last day when eggs were produced. However, no significant effects were found.

Plasma vitellogenin: Vitellogenin concentration in blood of male and female was analysed in experiment A. No induction of vitellogenin was found in experiment A (Figure 2 a, b).

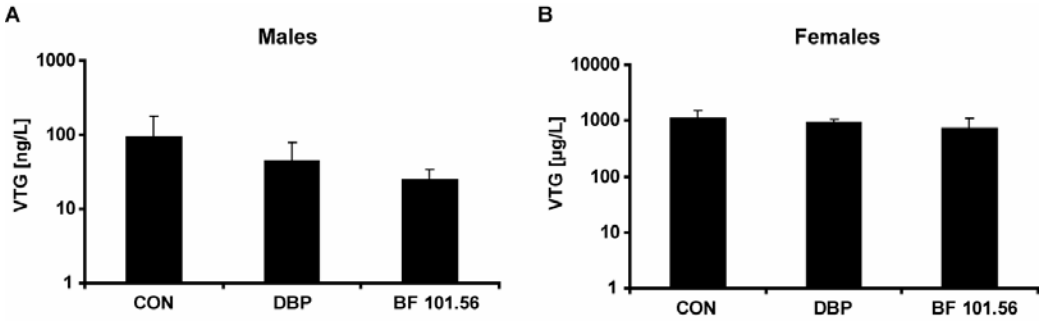
FAO activity: FAO activity expressed as nM DCF/min*mg protein in liver was measured in the male fish of experiment A, and in male and female fish of experiment C. A statistical analysis demonstrated that there was no difference in FAO activity between pond and labor reared fishes in experiment A. No statistically significant effects on FAO activity in fish were observed after 14 days of exposure to BF (Figure 3a). In contrast,

Table 3: Egg production and last day of egg laying (mean±SD) of fish exposed to clofibric acid in experiment C.

	Egg production	Last day of laying
CONTROL	6.3 ± 1.2	20.3 ± 1.5
1.07 mg/L CA	3.3 ± 2.0	17.3 ± 1.2
10.75 mg/L CA	3.3 ± 1.5	13.3 ± 2.9
108.91 mg/L CA	2.7 ± 2.5	13.3 ± 2.5

No significant effects were found, although a tendency to a decreased production of eggs occurs (p=0.052) at 1.07 mg/L CA and 10.75 mg/L CA.

Figure 2: Plasma vitellogenin concentrations in control fish (CON), the positive control dibutyl phthalate (0.5 mg/L) and fish exposed to 101.56 mg/L bezafibrate (BF). Plasma VTG of male (A) and females (B) as means ± SD. No significant induction of plasma VTG has been found both in male and female, p>0.05.



exposure to 108.91 mg/L CA for 21 days let to a significant increase in FAO activity in male fish (p=0.04) but this was not the case in female fish (Figure 3b). In male fish the effect was not completely dose-dependent. Even if fish exposed to 108.91 mg/l CA showed a statistically significant increase in the FAO activity compared to both control and 10.75 mg/L CA (p=0.041), this was not the case when compared to 1.07 mg/L CA (p>0.05). When male and female were pooled there was a significant increase in FAO activity of fish exposed to 108.91 mg/L CA compared to control (p=0.015) and to 10.75 mg/L CA (p=0.036). The activity of FAO of male fish exposed to 108.91 mg/L of CA was 1.5-times higher than that of control fish. In pooled male and female fish, this effect at 108.91 mg/L CA was 1.3 fold higher compared to control fish. Relative activities are shown in Table 4.

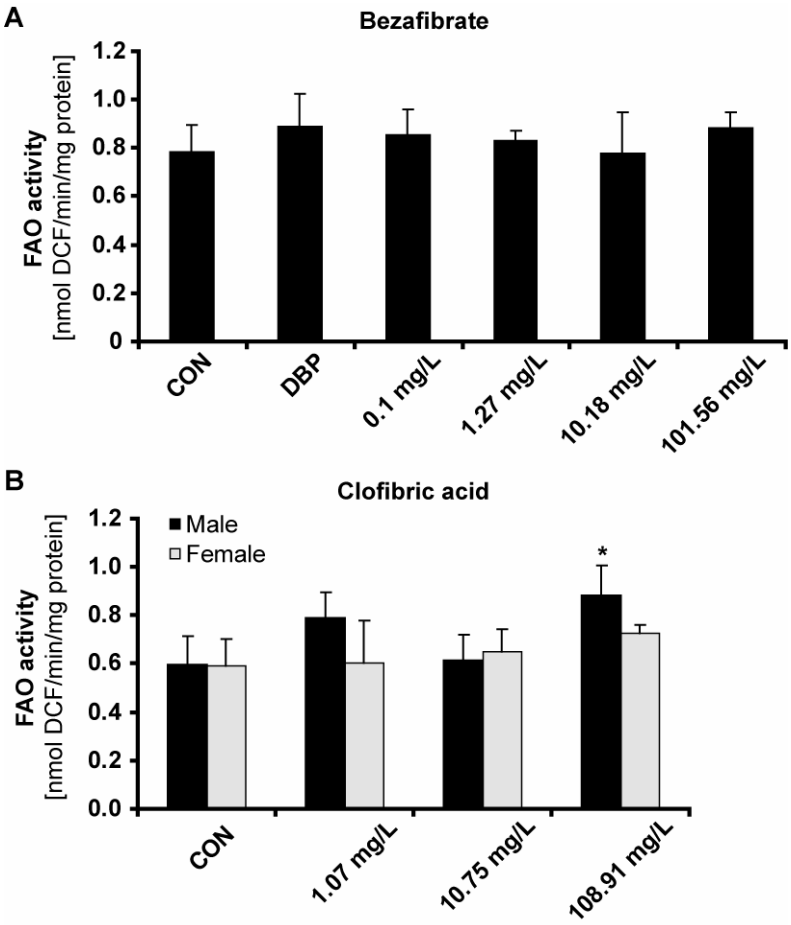
PPARα expression: PPARα expression was measured in fishes of experiment B and C. The expression was normalised for RNA concentration and the housekeeping gene β-actin. As shown in Figure 4a BF does not seem to affect the expression of PPARα (p>0.05) at the tested concentration of 106.7 mg/L BF. Males and female were analysed separately and in both cases, there was not a significant increase of PPARα

Table 4: Relative FAO activity in fish exposed to clofibric acid of experiment C.

	Relative FAO activity		
	Male	**Female**	**Male and Female**
CONTROL	1	1	1
1.07 mg/L CA	1.325	1.016	1.148
10.75 mg/L CA	1.032	1.099	1.080
108.91 mg/L CA	1.491*	1.221	1.256*

A significant increase in FAO activity was found in male fish exposed to 108.91 mg/L CA (*p=0.042).

Figure 3: FAO activity in liver of fish. Male fish exposed to bezafibrate of experiment A (A) and male and female fish exposed to clofibric acid of experiment C (B). Shown are means ± SD. FAO activity of male fish exposed to clofibric acid at 108.91 mg/L is significantly increased (*p<0.05).

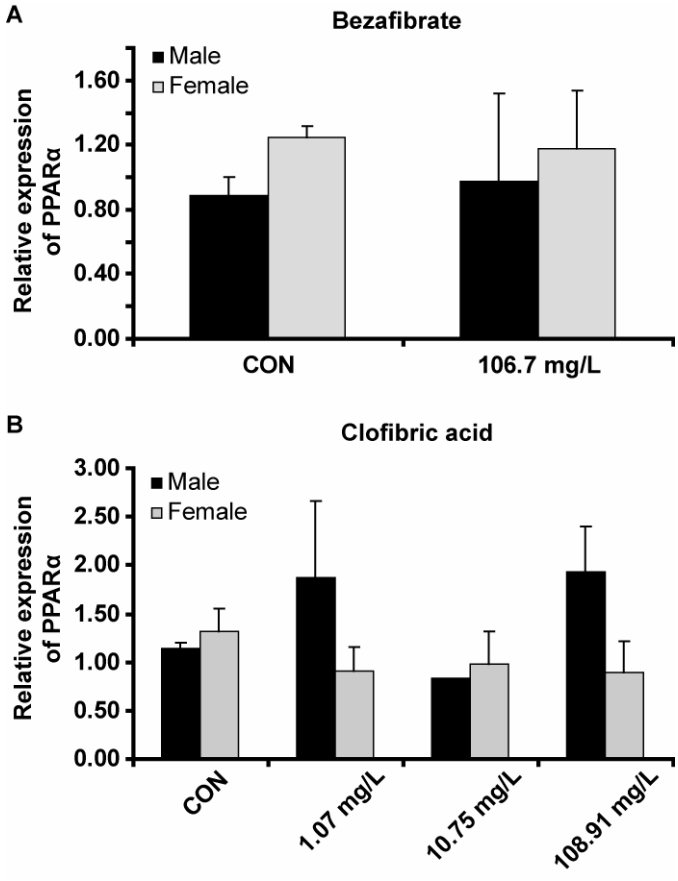


expression. Also for CA there was not a clear induction of PPARα expression. In Figure 4b PPARα expression of fish exposed to CA were shown for male and female separately. Males showed an increased expression at 1.07 and 108.81 mg/L CA, but this was not significant.

Discussion

In this study, effects of bezafibrate and clofibric acid have been assessed in fathead minnows after chronic exposure for 14 and 21 days. To our knowledge effects of these lipid lowering agents have not yet been investigated so far in fathead minnows, in particularly focussing on their mode of action. The blood lipid lowering agent bezafibrate and the parent compounds of clofibric acid are widely used in medicine to

Figure 4: PPARα expression in liver of male and female fish relative to housekeeping gene β-actin. (A) Fish exposed to bezafibrate of experiment B. (B) Fish exposed to clofibeic acid of experiment C. Data as means ± SD. No significant effects were found.



treat lipidemic disease such as hypercholesteremia and to prevent heart attack. These pharmaceuticals belong to the class of fibrates which are known to bind to peroxisome proliferating activating receptors (PPAR). These are nuclear receptors which enter the nucleus, heterodimerize with the RXR receptor and promote both the expression of different genes involved in lipid metabolisms, e.g. fatty acyl oxidase, and the proliferation of peroxisomes. Substances such as perfluoroctanoic acid and phthalates have been found to be PPAR activator (Ibabe *et al.* 2005) and exposure of aquatic organisms to such compounds showed increased FAO activity or PPAR expression. PPAR agonists may be also related to reproductive and developmental toxicity (Ackers *et al.* 2000).

Both bezafibrate and clofibric acid have been found in the environment in concentration that reach several µg/L in sewage treatment effluents and in surface water (Buser *et al.* 1998; Stumpf *et al.* 1996; Ternes 1998).

The data presented in this study indicate that bezafibrate does not seem to affect any of the parameter chosen at fairly high concentrations, which are orders of magnitudes higher than environmental concentration. For clofibric acid, however a small increase (1.5-fold) of the fatty acyl oxidase activity was found at 108.91 mg/L. Ortiz-Zarragoitia *et al.* (2004) found an increase in the FAO activity of fish exposed to perfluoroctanoic acid, which is also known to be a PPAR receptor agonist. Scarano (1994) investigated the effects on FAO activity on rainbow trout and medaka. The activity we found was lower compared to the activity measured by Scarano (1994). In fact, even in control fish we found, in both experiment, an activity, which was four times less (0.6-0.8 nmol/min*mg protein in our study compared to 2.5 nmol DCF/mg*protein in Scarano *et al.*). Scarano (1994) found an increase of FAO activity in rainbow trout, this effect appeared only if the data were analyzed in nmol DCF/mg liver. We decided that normalization on protein concentration was more reliable for our aim. Ruyter *et al.* (1997) analyzed effects on FAO activity of rainbow trout hepatoma cell lines exposed to bezafibrate and clofibric acid. These authors found a higher FAO activity, with values ranging from 2.5 to 4.35 for CA, and to 4.075 for BF compared to our data and they found a significant increase after exposure to both substances. In case of gemfibrozil FAO activity was not affected in fish (Mimeault *et al.* 2006). The results in our study for CA correspond to those found in an *in vitro* study with PLHC-1 cell lines, where an induction in FAO activity was found at 100 µM CA (Caminada *et al.* submitted). However, the induction found *in vitro* in PLHC-1 after exposure to BF was not confirmed in our *in vivo* study.

The increase in FAO activity by CA was not paralleled by an increase in PPARα expression. Whether this is a reflection of an early up-regulation of PPARα expression as *in vitr*o in PLHC-1 cells, followed subsequently by a down-regulation of this receptor, remains an open question. At the same time it is not known, whether the induction of this nuclear receptor and of regulatory proteins of fatty acid oxidation are subject to similar regulation processes and timing. It is also possible that, by increasing the sensitivity of the real-time method by using for example probes such as TaqMan smaller variations of PPARα may be detected.

PPAR receptors belong to DNA receptors, enter the nucleus when activated by a ligand and after dimerization with the RxR receptor. By binding to the DNA the

expression of different enzymes involved, among other cellular functions, in fatty oxidation, is enhanced. An increase in PPARγ expression was found by Ruyter (1997), where a 1.6 fold increase in PPARγ receptor was found after exposure to BF and CA in hepatoma cell lines of rainbow trout (Ruyter *et al.* 1997). On the other hand, Mimeault (2006) did not find any increase in the expression of PPARα in Goldfish after 14 and 28 days of exposure to 1.5 and 1.500 µg/L gemfibrozil. It is possible that a 14 to 21 days exposure to fibrates in fish is too long to detect an induction at receptor level. Support for this hypothesis is provided by our *in vitro* study (Caminada *et al.* submitted), in which an induction of PPARα and PPARγ was found in PLHC-1 cell lines after a short-term (6 hours), but not after a long-term (7 to 14 days) exposure to bezafibrate and clofibric acid. A comparison between *in vivo* and *in vitro* data both found in the literature and obtained in our laboratory seem to demonstrate that a first short term induction of PPARα may occur followed by a rapid recovery. The induction of enzyme seems, on the other hand, to had longer-lasting effect in the cells.

Another interesting result of the exposure to CA, was the decrease of egg production, even if this was not significant. This effect may be related to the known decrease on plasma testosterone of goldfish exposed to a drug of the fibrates family, gemfibrozil (Mimeault *et al.* 2005) and even on sperm amount and motility of fish exposed to CA (Runnalls *et al.* 2007). If the non significant decrease in egg production found in our experiment could be related to other reproductive effects such as sperms motility remains unclear and need to be further investigated. Whether indirect effects on reproduction of females occur via negative interaction with lipid metabolism remains open. This can only be investigated by pertinent reproductive experiments. The lack of egg production after exposure to BF in experiment A and B can be only explained as follows. For experiment A fish were almost two years old, and were previously used for breeding, which could explain the lack of reproduction in all groups. On the other hand, in experiment B the fish were slightly older than 6 months, and they could have been too young for reproduction. Vitellogenin was not induced in male or female fish exposed to BF.

None of the parameters analyzed in our study seemed to significantly affect fish exposed to BF and CA (except FAO activity at high CA concentration). Furthermore, the effects were found at several orders of magnitude higher concentration than found in the environment maximal concentration found in wastewater treatment plants

effluents are 4.6 µg/L and 1.6 µg/L for BF and CA, respectively (Buser *et al.* 1998; Ternes 1998). Therefore it seems that these two lipid-lowering agents do not pose a risk for fish with respect to the endpoints determined in our study related to the mode of action of these drugs. This does not necessary rule out the possibility of unexpected effects that may occur as in case of other pharmaceuticals (Runnalls *et al.* 2007).

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CHAPTER 7

General Discussion

General discussion

The results of the different studies in chapter two to six are discussed in detail in the corresponding chapters. Here, the findings are summarized and discussed in general terms including their relevance within the field of ecotoxicology and towards the risk assessment of pharmaceuticals in the environment.

This dissertation is focused on effects of human pharmaceuticals towards aquatic organisms. Residues of pharmaceuticals are regularly found in surface waters in the range of ng/L to µg/L. At polluted sites, aquatic organisms are exposed during their whole lifetime to a variety of micro-pollutants, but the risks of permanent exposure to environmental xenobiotics are largely unknown. Pharmaceuticals are designed to exhibit specific modes of action at often low concentrations on target biomolecules in humans. Current data indicate that most of the targets are also present in lower organisms. Therefore, pharmaceuticals may have similar modes of action and effects in fish as in humans.

So far, most ecotoxicological studies have focused on established endpoints such as acute toxicity, effects on the detoxification system (phase I and phase II), induction of heat shock proteins, etc. This approach is not sufficient enough for the investigation of potential chronic effects of substances interacting specifically with defined targets like receptors, enzymes and transporters in the cell. Therefore, a mechanism-based approach is necessary. As only little is known about the presence and function of these targets in aquatic organisms, they have to be found and characterized first.

In this dissertation, several studies are performed to elucidate cellular mechanisms of pharmaceuticals in fish. The cytotoxicity of environmentally important pharmaceuticals in *in vitro* model systems are assessed and correlated with results from *Daphnia magna* and fish. Further, we succeeded in selecting a doxorubicin-resistant PLHC-1 subclone (PLHC-1/dox) characterized in the overexpression of P-glycoprotein (ABCB1). This transporter plays a crucial role in detoxification processes and its modulation can essentially change the intracellular concentration of compounds. The data demonstrate the presence of a classical multidrug resistance phenotype in a non-mammalian cell system. A subsequent study shows that several pharmaceuticals affect the multidrug resistance mechanism in PLHC-1/wt and PLHC-1/dox cells. Finally, we

demonstrated the presence of the receptor based PPAR-system in PLHC-1 cells and analyzed effects mediated by fibrates on the expression of these receptors as well as on the activity of a peroxisomal enzyme. In the following a short summary of the different achievements with the most important conclusions are given.

Cytotoxicity of pharmaceuticals

We evaluated the *in vitro* cytotoxicity of 34 common pharmaceuticals from different classes and with different modes of action using the mitochondrial MTT reduction and NR uptake assays in the two fish cell lines, PLHC-1 and RTG-2. Cytotoxicity was found for 21 pharmaceuticals with no significant difference between the MTT and NR assays. The comparison of the cell lines revealed that PLHC-1 cells were slightly more sensitive to cytotoxic effects than the RTG-2 cells. We demonstrated that the cytotoxicity of pharmaceuticals correlated with their LogD values at physiological conditions (pH 7.0). A correlation between the *in vitro* data and *in vivo* data was found for *Daphnia magna*, but not for fish due to insufficient and heterogeneous data.

The findings provide an indication that *in vitro* cytotoxicity assays with fish cell lines are suitable for the first screening of the acute *in vivo* toxicity of pharmaceuticals and possibly other compounds, thereby contributing to the reduction of *in vivo* experiments. The EC50-values of the pharmaceuticals showing a correlation with their LogD indicates that the cytotoxic effect is probably due to non-specific toxicity or narcosis. However, some pharmaceuticals like doxorubicin and fluoxetine did not correlate with their LogD value pointing at a specific mode of toxic action.

Effects of pharmaceuticals on the MDR/MXR mechanism

We were able to select a highly doxorubicin-resistant PLHC-1 subclone (PLHC-1/dox) by culturing wild type cells in the presence of doxorubicin. We demonstrate that a specific, P-gp1 mediated doxorubicin resistance mechanism is present in the PLHC-1 fish cell line. In addition, the fact that low micromolar concentrations of specific inhibitors may completely reverse a highly expressed doxorubicin resistance points to the fragility of P-gp1 mediated MXR defence mechanism in fish. The data demonstrate for the first time the presence of a classical multidrug resistance phenotype in non-mammalian cells.

The aim of our subsequent work was the analysis of a series of pharmaceuticals for their potential to interact and modulate the activity of xenobiotic efflux transporters from the ABCB and ABCC sub-family in the PLHC-1 fish cell line (PLHC-1/wt) and the doxorubicin resistant PLHC-1 subclone (PLHC-1/dox). Our work revealed significant inhibitory effects of environmentally relevant pharmaceuticals on ABC transporters, demonstrating that this class of compounds interacts with the MDR/MXR mechanism in fish. Our findings correspond well with data from mammalian systems indicating that the specificity and roles of the efflux transporters are similarly in fish.

Detection and characterization of PPARs in PLHC-1 cells and fathead minnows

This piece of work aimed at the detection and characterisation of the receptor-based PPAR-system in the fish cell line PLHC-1. We found all three PPARs (PPARα, PPARβ and PPARγ) present in the cells as well as the retinoid X receptor (RxR) that forms a heterodimer with the activated PPAR-receptor, and thus, is necessary for a response. The sequences showed high similarities to other known sequences in fish, especially with *Dicentrarchus labrax* and *Sparus aurata*. In expression analysis, the highest expression level was found for PPARα followed by PPARγ and PPARβ. Upon exposure to different fibrates for 6 h, the expression level of PPARα and PPARγ was induced up to ten-fold. Further, an increased activity of the peroxisomal enzyme fatty acyl oxidase was found that is involved in the β-oxidation pathway of fatty acids and that is regulated by PPARα. These results demonstrate for the first time the presence and function of PPARs in a fish cell line.

Our interest in the following study focused on biochemical effects on the liver of fish after 14 and 21 days exposure to bezafibrate and clofibric acid, respectively, focussing in particularly on PPARs and peroxisomal enzymes. In addition we monitored reproductive parameter such as egg production and vitellogenin induction to investigate the hypothesis whether the effects of these human pharmaceuticals may be also linked to reproductive toxicity. Male fathead minnows exposed to clofibric acid showed an increase in FAO activity at the highest concentration only; this effect was not found in female and could not be related to an increase in PPARα expression. This indicates that *in vitro* and *in vivo* results are comparable, but only to a certain extent. Effect concentrations and even mechanistic parameters (induction of PPARα) diverge due to different exposures and time-scales. In addition, a non-significant decrease in egg production after 21 days of exposure to clofibric acid was found.

A comprehensive evaluation of the potential risk of pharmaceuticals to the (aquatic) environment demands a mechanism based approach to account the specific modes of action of compounds. Pharmaceuticals exhibit often important side effects besides their therapeutic properties. Unexpected effects in lower organisms may also appear due to biological differences in pharmacodynamcis, pharmacokinetics and physiology. Only chronic toxicity investigations using more specific toxicity parameters will lead to a more meaningful ecological risk assessment. Regarding the amount of substances to be evaluated and the many different targets to be taken into account, *in vitro* studies of pharmaceuticals are important for screening, elucidating the modes of action in non-target organisms, and designing specific *in vivo* studies.

A comparison of effects of fibrates in the PLHC-1 cells with effects *in vivo* was performed. Pronounced effects on the expression of fibrates as well as on the FAO-activity were found *in vitro*, whereas *in vivo* only an effect on the FAO-activity was measured. These studies reveal two important aspects: first, we could prove our mechanism-based hypothesis that pharmaceuticals act on the same targets as in mammals; in our case, fibrates are ligands of PPARα in PLHC-1 cells. Second, the cell line was a sensitive and appropriate tool to detect the receptors and to establish assays for the investigations of their effects. In contrast, standardized protocols for *in vivo* investigations are not found to be sensitive enough to find specific effects. Instead, the protocols have to be adapted in order to find both relevant exposure times and endpoints. The *in vitro* system allows a better characterization of the involvement of PPARs, whereas the *in vivo* study actually represents a more realistic exposure situation. Knowledge from *in vitro* studies helps to design the more complex *in vivo* experiments.

Knowledge on targets like receptors with which pharmaceuticals interact as well as toxicological mechanisms in aquatic organisms is lacking to a large extent. Therefore, such knowledge is gathered in this dissertation as a basis for further toxicological studies. In the field of multidrug resistance/multixenobiotc resistance, we did the first step towards the development of more appropriate model systems by selecting an ABCB1 overexpressing subclone. It is the first time that a specific P-gp1 mediated doxorubicin resistance mechanism has been shown to be present in cells derived from

an aquatic organism. In ongoing studies, we are trying to select further subclones overexpressing other kinds of transporters like multidrug resistance-related proteins (MRP1, MRP3) and breast cancer resistance protein (BCRP) involved in the efflux of xenobiotics. The successful selection of additional subclones will allow a better understanding of the biochemical mechanisms involved in multidrug resistance mechanisms in aquatic organisms and facilitate the characterisation of the role and specificities of these transporters in aquatic organisms. Subject of forthcoming studies is also the cloning, transfection and expression of selected fish ABC-transporters in appropriate systems like insect cells allowing more specific molecular-biological investigations.

Peroxisome proliferator-activated receptors (PPARs) play important functions during the development of an organism, but also in the receptor mediated regulation of the fatty acid metabolism, lipid homeostasis, differentiation of adipocytes and epithelial tissues. Further, in mice and rats, but not in humans, it has been shown that PPARα is involved in the hepatocarcinogenesis mediated by fibrates. It is not known whether carcinogenesis mediated by PPARα activation can occur in fish. The detection and functional characterisation of PPARs in PLHC-1 cells allows further investigations of the processes regulated by PPARs in fish. Together with the knowledge of MDR mechanism and induction of CYP1A1 in PLHC-1 cells there are currently several elements that may have implications on carcinogenesis. As xenobiotics having the potential to interact with targets involved in carcinogenic processes are present in the aquatic environment, investigations of these processes in fish may gain more importance in future research. The PLHC-1 cell line could represent a reliable tool for such studies.

In conclusion, this dissertation gives new insights into the interaction of pharmaceuticals on the cellular level with different targets like ABC-transporters and PPARs. The PLHC-1 cells are found to be a reliable *in vitro* model system for the investigation of biochemical and molecular-biological mechanisms in fish. Our findings that different efflux transporters as well as the three PPAR- and the RxR-receptor are present and functional in this cell line - in addition to the already characterized function of the aryl hydrocarbon (Ah) receptor involved in the regulation of CYP1A expression – indicates that many of the metabolic pathways still work similarly as *in vivo*. Therefore, this cell line is very promising in further research on the investigation of the modes of

action of environmentally relevant pharmaceuticals and other xenobiotics on the cellular level.

In terms of environmental practice, cytotoxicity assays in fish cell lines can be a valuable tool in the risk assessment to estimate and rank the acute toxicity of compounds in order to minimize acute toxicity tests *in vivo*. They may be included in a tiered approach to assess the ecotoxicity of pharmaceuticals, but may also gain importance in the toxicity assessment of chemicals in the future strategy of the European Union within the REACH concept. However, for this purpose further validation is needed in ring studies with a broader set of compounds and in respect to the correlation with *in vivo* data.

Cytotoxicity in fish cells occured at concentrations of about 1'000'000 higher than environmental concentrations. Moreover, effects of single compounds on efflux transporters and PPARα induction in cell lines and fish occurred at concentrations that are a factor 1000 higher than ambient levels. However, mixtures of compounds acting on the same targets usually exhibit an additive nature, e.g. all individual components contribute to the overall effect of a mixture. This implies that the overall effect exceeds the highest individual effect of the mixture's components. Further, as found for the efflux transporters, interactions of certain pharmaceuticals with these transporters leads to inhibition of efflux activity, and therefore compound mixtures may increase the toxicity to environmental pollutants resulting in a more than additive or synergistic phenomenon. These considerations are of particular importance for the environmental hazard and risk assessment of pharmaceuticals, because it indicates that concentrations of single chemicals that show no effect when applied singly may provoke (substantial) effects when acting in combination.

Outlook

Our studies reveal new insights towards a mechanism based approach in the evaluation of ecotoxicological effects of pharmaceuticals in *in vitro* systems for aquatic organisms. Our mechanism-based research focused on the characterisation of multidrug resistance proteins and PPARs and showed that pharmaceuticals interact

with these targets. This allows a better understanding of these processes and mechanisms on the cellular level.

The effects found in our *in vitro* studies occurred only at concentrations of about a factor 1000 higher than environmental concentrations, but they may contribute to the overall toxicity of a mixture. For further studies aimed at a better risk assessment of these compounds we propose the following issues to be addressed:

- What are the specific interactions of other environmental pollutants (including pharmaceuticals) towards the investigated targets?
- What other cellular targets are affected by pharmaceuticals?
- What effects do compound mixtures and environmental samples exhibit on these targets?
- How do *in vitro* effects in fish cells correlate with *in vivo* effects in fish?

These questions are addressed in ongoing projects in our laboratory. The results from the *in vitro* assays of this dissertation are the basement for the design of further *in vivo* investigations. They will reveal whether *in vitro* effects in fish cells are less (or more) sensitive than effects *in vivo*. Moreover, this will indicate what the cellular effects found will signify for aquatic organisms. The *in vitro* system used in this study has the disadvantage that it is derived from a rarely used fish in ecotoxicology (topminnow, *Poeciliopsis lucida*). So far, *in vivo* experiments in our laboratory were performed with fathead minnow (*Pimephales promelas*). However, the best characterized and most often used model system for aquatic organisms represents the zebrafish (*Danio rerio*). It is widely used by developmental geneticists and it was the first aquatic organism whose genome was sequenced. Therefore, zebrafish will be used in our laboratory for future *in vivo* investigations. In order to expand mechanism based investigations, this model system can further be used for toxicogenomic analysis using microarray techniques.

Pharmaceuticals represent only a small fraction of numerous different xenobiotics found in the environment. Further studies should focus on the specificity of the targets characterized in this dissertation towards other environmental pollutants. During this dissertation, different mechanisms were investigated using single compounds or dual mixtures. However, environmental samples usually contain a complex mixture of different compounds that may be able to increase the biological potency. To address

this question, effects of compound mixtures and of environmental samples on specific targets should be investigated in future studies.

Appendix 1

Ecotoxicology of human pharmaceuticals

Karl Fent[a,b], Anna A. Weston [a,c] and Daniel Caminada [a,d]

[a] University of Applied Sciences Basel, Institute of Environmental Technology, St. Jakobs-Strasse 84, CH-4132 Muttenz, Switzerland

[b] Swiss Federal Institute of Technology (ETH), Department of Environmental Sciences, CH-8092 Zürich

[c] Springborn Smithers Laboratories Europe AG, Seestrasse 21, CH-9326 Horn, Switzerland

[d] University of Zürich, Institute of Plant Biology, Limnology, Seestrasse 187, CH-8802 Kilchberg, Switzerland

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Abstract

Low levels of human medicines (pharmaceuticals) have been detected in many countries in sewage treatment plant (STP) effluents, surface waters, seawaters, groundwater and some drinking waters. For some pharmaceuticals effects on aquatic organisms have been investigated in acute toxicity assays. The chronic toxicity and potential subtle effects are only marginally known, however. Here, we critically review the current knowledge about human pharmaceuticals in the environment and address several key questions. What kind of pharmaceuticals and what concentrations occur in the aquatic environment? What is the fate in surface water and in STP? What are the modes of action of these compounds in humans and are there similar targets in lower animals? What acute and chronic ecotoxicological effects may be elicited by pharmaceuticals and by mixtures? What are the effect concentrations and how do they relate to environmental levels? Our review shows that only very little is known about long-term effects of pharmaceuticals to aquatic organisms, in particular with respect to biological targets. For most human medicines analyzed, acute effects to aquatic organisms are unlikely, except for spills. For investigated pharmaceuticals chronic lowest observed effect concentrations (LOEC) in standard laboratory organisms are about two orders of magnitude higher than maximal concentrations in STP effluents. For diclofenac, the LOEC for fish toxicity was in the range of wastewater concentrations, whereas the LOEC of propranolol and fluoxetine for zooplankton and benthic organisms were near to maximal measured STP effluent concentrations. In surface water, concentrations are lower and so are the environmental risks. However, targeted ecotoxicological studies are lacking almost entirely and such investigations are needed focusing on subtle environmental effects. This will allow better and comprehensive risk assessments of pharmaceuticals in the future.

Key words: Pharmaceuticals – Ecotoxicological Effects – Environmental Toxicity – Chronic Effects – Environmental Risk Assessment

Introduction

It came as a surprise when an unusually high death rate among three species of vulture in India and Pakistan was reported in 2004 to be caused by diclofenac, a widely used analgesic and antiinflammatory drug (Oaks *et al.* 2004). The Oriental white-backed vulture (Gyps bengalensis) is one of the most common raptors in the Indian subcontinent and a population decline of >95% makes this species as being critically endangered. Whereas a population decline has started in the 1990s, recent catastrophic declines also involve Gyps indicus and Gyps tenuirostris across the Indian subcontinent (Prakash *et al.* 2003; Risebrough 2004). High adult and subadult mortality and resulting population loss is associated with renal failure and visceral gout, the accumulation of uric acid throughout the body cavity following kidney malfunction. A direct correlation between residues of diclofenac and renal failure was reported both by experimental oral exposure and through feeding vultures diclofenac-treated livestock. Hence, the residues of diclofenac were made responsible for the population decline (Oaks *et al.* 2004). This drug has recently come into widespread use in these countries as a veterinary medicine, but is also widely used as in human medicine since the 1970s. Vultures are natural scavengers feeding on carrion of wildlife and domestic livestock and cattle. The three vulture species continue to decline in Pakistan, India, Bangladesh and southern Nepal. Apart from this severe case, never having been anticipated, potential ecotoxicological effects of drug residues in the environment on wildlife are largely unknown.

Pharmaceuticals are a class of emerging environmental contaminants that are extensively and increasingly being used in human and veterinary medicine. These chemicals are designed to have a specific mode of action, and many of them for some persistence in the body. These features among others make pharmaceuticals to be evaluated for potential effects on aquatic flora and fauna. The current investigations are mainly driven by advances in environmental residue analysis, particularly after the establishment of chemical analysis methods able to determine more polar compounds such as liquid chromatography-tandem mass spectrometry, which allows the identification of trace quantities of polar organic pollutants without derivatization (Daughton and Ternes 1999; Kolpin *et al.* 2002; Kümmerer 2004). Accordingly, many environmental analyses have been performed in various countries, which are summarized by various reports (e.g. Daughton and Ternes 1999; e.g. Halling-Sorensen *et al.* 1998; Kümmerer 2004). These monitoring studies demonstrate that drug residues in treated wastewater and surface water are very widespread.

In contrast, only little is known about ecotoxicological effects of pharmaceuticals on aquatic and terrestrial organisms and wildlife, and a comprehensive review on ecotoxicological effects is lacking. Aquatic organisms are particularly important targets, as they are exposed via wastewater residues over their whole life. Standard acute ecotoxicity data have been reported

for a number of pharmaceuticals, however, such data alone may not be suitable for specifically addressing the question of environmental effects, and subsequently in the hazard and risk assessment (Fent 2003). The current lack of knowledge holds in particular for chronic effects that have only very rarely been investigated. In spite of the sizeable amounts of human drugs released to the environment, concise regulations for ecological risk assessment are largely missing. Only in the last few years, regulatory agencies have issued detailed guidelines on how pharmaceuticals should be assessed for possible unwanted effects on the environment. The first requirement for ecotoxicity testing as a prerequisite for registration of pharmaceuticals was established in 1995 according to the European Union (EU) Directive 92/18 EEC and the corresponding “Note for Guidance” (EMEA 2005) for veterinary pharmaceuticals. The European Commission released a draft guideline (Directive 2001/83/EC) specifying that an authorization for a medicinal product for human use must be accompanied by an environmental risk assessment (EMEA 2005). The U.S. Food and Drug Administration (FDA) published a guidance for the assessments of human drugs; according to this, applicants in the U.S.A. are required to provide an environmental assessment report when the expected introduction concentration of the active ingredient of the pharmaceutical in the aquatic environment is ≥ 1 µg/L (FDA-CDER 1998), which corresponds to about 40 tons as a trigger level. In contrast, environmental assessments of veterinary pharmaceuticals is required by the U.S. FDA since 1980 (Boxall *et al.* 2003).

The objective of our paper is to compile and critically review the present knowledge about the environmental occurrence and fate of human pharmaceuticals in the aquatic environment, to discuss potential mechanisms of action based on knowledge from mammalian studies, and to describe the acute and chronic ecotoxicological effects on aquatic organisms. We also identify major gaps in the current knowledge and future research needs. We concentrate on pharmaceuticals used in human medicine, some of which are also applied in veterinary medicine, thereby focusing on environmentally important compounds belonging to different drug categories, namely non-steroidal antiinflammatory drugs, beta-blockers, blood lipid lowering agents, cancer therapeutics and neuroactive compounds. These classes differ for their modes of actions and were chosen because of their consumption volumes, toxicity and persistence in the environment. We will not address the environmental effects of antibiotics and biocides (Daughton and Ternes 1999; Halling-Sorensen *et al.* 1998; Hirsch *et al.* 1999), hormones (used in contraceptives and in therapy) (Damstra *et al.* 2002) and special veterinary pharmaceuticals (Boxall *et al.* 2003; Montforts *et al.* 1999) as the cited reports provide detailed information.

The current knowledge indicates that residues of pharmaceuticals at trace quantities are widespread in aquatic systems. Pharmaceuticals in the environment are suggested to pose only a low risk for acute toxicity. For chronic effects, the situation may be different, but there is a considerable lack of information. Investigation of multigenerational life-cycle effects or at various

life stages is lacking, although many aquatic organisms are exposed for their entire life. There is a need to focus on long-term exposure assessment regarding specific modes of action of pharmaceuticals to better judge the implications of pharmaceutical residues in aquatic systems. Only after filling these gaps, more reliable environmental risk assessments with much lower uncertainty can be performed.

Sources

The consumption of pharmaceuticals is substantial. In the European Union (EU) about 3'000 different substances are used in human medicine such as analgesics and antiinflammatory drugs, contraceptives, antibiotics, beta-blockers, lipid regulators, neuroactive compounds and many others. Also a large number of pharmaceuticals are used in veterinary medicine, among them antibiotics and antiinflammatory. Sales figures are relatively high as reported for several countries (Table 1). In England, Germany and Australia, the amounts for the most frequently used drugs are in the hundreds of tons per year (Huschek *et al.* 2004; Jones *et al.* 2002; Oaks *et al.* 2004). The pattern of consumed pharmaceuticals for the different countries is not identical and some drugs may be forbidden or replaced by related drugs. However, as listed in Table 1, some drugs are regularly documented within the most frequently applied range: the class of non-steroidal antiinflammatory drugs (NSAID) including acetylsalicylic acid (e.g. 836 t in Germany in 2001), paracetamol (e.g. 622 t in Germany in 2001), ibuprofen (e.g. 345 t in Germany in 2001), naproxen (e.g. 35 t in England in 2000) and diclofenac (86 t in Germany in 2001), the oral antidiabetic metformin (e.g. 517 t in Germany 2001) and the antiepileptic carbamazepine (e.g. 88 t in Germany 2001). Data representing the annual sales or consumptions include mainly prescribed drugs, some include also sales over-the-counter, some a mixture of both, and internet sales are not included. Therefore, the real amounts of applied drugs is uncertain, but probably significantly higher for some of the pharmaceuticals reported than the figures in Table 1. Figuring out the annual consumption of a certain drug is difficult and often based on estimates. For example, based on sales, estimates of the U.S. production of the antiepileptic carbamacepine (which is also used for other treatments) ranged from 43 t in 2000 to 35 t in 2003 (Thaker 2005).

Pharmaceuticals are excreted after application in their native form or as metabolites and enter aquatic systems via different ways. The main pathway from humans is ingestion following excretion and disposal via wastewater. Municipal wastewater is therefore the main route that brings human pharmaceuticals after normal use and disposal of unused medicines into the environment. Hospital wastewater, wastewater from manufacturers and landfill leachates (Lilius *et al.* 1994) may contain significant concentrations of pharmaceuticals. Pharmaceuticals not readily degraded in the sewage treatment plant (STP) are being discharged in treated effluents

Table 1
Annual consumption of different classes of prescribed drugs for different countries

Compounds	Germany 1999[a]	Germany 2000[a]	Germany 2001[a]	Austria 1997[b]	Denmark 1997[c]	Australia 1998[d]	England 2000[e]	Italy 2001[f]	Switzerland 2004[g]
Analgesics, antipyretics and anti-inflammatory									
Acetylsalicylic acid	902.27 (1)	862.60 (1)	836.26 (1)	78.45 (1)	0.21 (7)	20.4 (9)			43.80 (3)
Salicylic acid	89.70 (12)	76.98 (17)	71.67 (17)	9.57 (11)					5.30 (6)
Paracetamol	654.42 (2)	641.86 (2)	621.65 (2)	35.08 (2)	0.24 (6)	295.9 (1)	390.9 (1)		95.20 (1)
Naproxen				4.63 (16)		22.8 (7)	35.07 (12)		1.70 (12)
Ibuprofen	259.85 (5)	300.09 (5)	344.89 (5)	6.7 (13)	0.03 (19)	14.2 (13)	162.2 (3)	1.9 (15)	25.00 (4)
Diclofenac	81.79 (16)	82.20 (14)	85.80 (14)	6.14 (15)			26.12 (16)		4.50 (7)
β-Blocker									
Atenolol							28.98 (13)	22.07 (4)	3.20 (9)
Metoprolol	67.66 (18)	79.15 (16)	92.97 (11)	2.44 (20)					3.20 (10)
Antilipidemic									
Gemfibrazol						20 (10)			0.399 (18)
Bezafibrate				4.47 (17)				7.60 (8)	0.757 (15)
Neuroactive									
Carbamazepine	86.92 (13)	87.71 (13)	87.60 (12)	6.33 (14)		9.97 (18)	40.35 (8)		4.40 (8)
Diazepam					0.21 (8)				0.051 (21)
Antiacidic									
Ranitidine	85.41 (15)	89.29 (12)	85.81 (13)			33.7 (5)	36.32 (10)	26.67 (3)	1.60 (13)
Cimetidine							35.65 (11)		0.063 (20)
Diuretics									
Furosemide					3.74 (1)			6.40 (19)	1.00 (14)
Sympatomimetika									
Terbutalin					0.46 (3)				0.0099 (23)
Salbutamol					0.17 (9)				0.035 (22)
Various									
Metformin	368.01 (4)	433.46 (4)	516.91 (3)	26.38 (3)		90.9 (2)	205.8 (2)		51.40 (2)
Estradiol					0.12 (13)				
Iopromide	64.93 (19)	63.26 (19)	64.06 (19)						6.90 (5)

For every country a top 20 sold-list is taken into account. Data in bracket represent the position in the ranking list within a country. Data are in t/year.

[a] Huschek et al. (2004).
[b] Sattelberger (1999).
[c] Stuer-Lauridsen et al. (2000).
[d] Khan and Ongerth (2004).
[e] Jones et al. (2002).
[f] Calamari et al. (2003).
[g] ©IMS Health Incorporated or its affiliates. All rights reserved. MIDAS–02/03/05.

resulting in the contamination of rivers, lakes, estuaries and rarely, groundwater and drinking water. Where sewage sludge is applied to agricultural fields, contamination of soil, run-off into surface water but also drainage may occur. In addition, veterinary pharmaceuticals may enter aquatic systems via manure application to fields and subsequent runoff, but also via direct application in aquaculture (fish farming). Of environmental concern is not necessarily a high production volume of a certain pharmaceutical per se, but the environmental persistence and critical biological activity (e.g. high toxicity, high potency for effects on biological key functions such as reproduction). As exemplified by the synthetic steroid hormones in contraceptive pills, such as 17□-ethinylestradiol (EE2), the annual production lies in a couple of hundreds kilograms per year in the EU, yet it is extremely potent, quite persistent in the environment and shows estrogenic activity in fish at 1-4 ng/L, or lower. Hence, pharmaceuticals having environmental relevance share the following properties: often, but not always, high production volume combined with environmental persistence and biological activity, mainly after long-term exposure.

Fate in the environment

The behaviour and fate of pharmaceuticals and their metabolites in the aquatic environment is not well known. The low volatility of pharmaceuticals indicates that distribution in the environment will occur primarily through aqueous transport, but also via food chain dispersal. In wastewater treatment, two elimination processes are generally important: adsorption to suspended solids (sewage sludge) and biodegradation. Adsorption is dependent on both hydrophobic and electrostatic interactions of the pharmaceutical with particulates and microorganisms. Acidic pharmaceutical such as the NSAID acetylsalicylic acid, ibuprofen, fenoprofen, ketoprofen, naproxen, diclofenac and indomethacin having pKa values ranging from 4.9 to 4.1, as well as clofibric aid, bezafibrate (pKa 3.6) and gemfibrozil occur as ion at neutral pH, and have little tendency of adsorption to the sludge. But adsorption increases with lower pH. At neutral pH, these negatively charged pharmaceuticals therefore occur mainly in the dissolved phase in the wastewater. For these compounds and the antitumor agent ifosfamide sorption by non-specific interactions seems not to be relevant (Buser *et al.* 1998b; Kümmerer 2004). In general, sorption of acidic pharmaceuticals to sludge is suggested to be not very important for the elimination of pharmaceuticals from wastewater and surface water. Therefore, levels of pharmaceuticals in digested sludge and sediments are suggested to be relatively low, as was demonstrated in several monitoring studies (Daughton and Ternes 1999; Urase and Kikuta 2005). However, basic pharmaceuticals and zwitterions can adsorb to sludge to a significant extent, as has been shown for fluoroquinolone antibiotics (Golet *et al.* 2002). For the hydrophobic EE2 (log K_{ow} 4.0) sorption to sludge is likely to play a role in the removal from

wastewater. Degradation in sludge seems not significant. As a consequence, EE2 occurs in digested sludge, where concentrations of 17 ng/g were reported (Temes *et al.* 2002).

In case a pharmaceutical is occurring mainly in the dissolved phase, biodegradation is suggested to be the most important elimination process in wastewater treatment. It can occur either in aerobic (and anaerobic) zones in activated sludge treatment, or anaerobically in sewage sludge digestion. In general, biological decomposition of micro-pollutants including pharmaceuticals increases with increase in hydraulic retention time and with age of the sludge in the activated sludge treatment. For example, diclofenac was shown to be significantly biodegraded only when the sludge retention time was at least 8 days (Kreuzinger *et al.* 2004). In contrast, data from Metcalfe et al. (2003) indicate that the neutral drug carbamazepine, which is hardly biodegradable, is only poorly eliminated (normally less than 10%), independent from hydraulic retention times. Pharmaceuticals are often excreted mainly as non-conjugated and conjugated polar metabolites. Conjugates can, however, be cleaved in sewage treatment plants (STP), resulting in the release of active parent compound as shown for estradiol (Daughton and Ternes 1999; Panter *et al.* 1999), and the steroid hormone in the contraceptive pill, 17α-etinylestradiol (D'Ascenzo *et al.* 2003).

Studies on the elimination rates during the STP process are mainly based on measurements of influent and effluent concentrations in STPs, and they vary according to the construction and treatment technology, hydraulic retention time, season and performance of the STP. Some studies (Carballa *et al.* 2004; Daughton and Ternes 1999; Stumpf *et al.* 1999) indicate elimination efficiencies of pharmaceuticals to span a large range (0-99%). The average elimination for specific pharmaceuticals varied from only 7-8% for carbamazepine (Clara *et al.* 2004; Daughton and Ternes 1999; Heberer 2002) up to 81% for acetylsalicylic acid, 96% for propranolol, and 99% for salicylic acid (Daughton and Ternes 1999; Heberer 2002). Lowest average removal rates were found for diclofenac (26%), the removal of bezafibrate was 51%, but varied significantly between STPs, and high removal rates were found for naproxen (81%) (Lindqvist *et al.* 2005). Table 2 shows that removal rates are variable, even for the same pharmaceutical between different treatment plants. Very high total elimination of 94-100% of ibuprofen, naproxen, ketoprofen and diclofenac was found in 3 STP in the U.S.A. (Hollert *et al.* 2000). Efficient removal took place mainly in the secondary treatment step (51-99% removal), whereas in the primary treatment only 0-44% were removed. X-ray contrast media (diatrizoate, iopamidol, iopromide, iomeprol), to the contrary, were not significantly eliminated (Daughton and Ternes 1999). Also, the anticancer drug tamoxifen (antiestrogen) was not eliminated (Roberts and Thomas 2005). This variation in elimination rates is not surprising, since pharmaceuticals form a heterogeneous group consisting of compounds with diverse chemical properties. Independent from the chemical characteristics of the compounds, the efficiencies of various STPs also vary for the same compound due to their equipment and treatment steps but also to

Table 2
Influent and effluent concentrations and removal efficiency in sewage treatment plants (different equipment, different countries, sampling in different seasons)

Compound	Influent concentration (μg/L)	Effluent concentration (μg/L)	Maximal removal (%)	Reference
Analgesics and antiinflammatory drugs				
Acetylsalicylic acid	3.2	0.6	81	Ternes et al. (1999)
Salicylic acid	57	0.05	99	Metcalfe et al. (2003a)[a]
	330	3.6		Carballa et al. (2004)
Dextropropoxyphene	0.03	0.06	0	Roberts and Thomas (2005)[a]
Diclofenac	3.0	2.5	17	Heberer (2002)
	n.r.	n.r.	69	Ternes (1998)[b]
	0.33–0.49	n.r.	75 (10–75)	Andreozzi et al. (2003a)[c]
	[5]	[1.5]	53–74	Strenn et al. (2004)[a]
	1.3	n.r.		Metcalfe et al. (2003a)[a]
	0.47–1.9	0.31–0.93		Buser et al. (1998b)
	2.8	1.9	23 ± 30	Quintana et al. (2005)[b]
	0.4–1.9	0.4–1.9	0	Tauxe-Wuersch et al. (2005)[c]
	0.35 ± 0.1	0.17–0.35	9–60	Lindqvist et al. (2005)[c]
	1.0	0.29	71	Roberts and Thomas (2005)[a]
Ibuprofen	3		96	Buser et al. (1999)
	38.7	4	>90	Metcalfe et al. (2003a)[a]
	9.5–14.7	0.01–0.02	99	Thomas and Foster (2004)
	[0.54]	[0.08–0.28]	22–75 99 (52–99)	Andreozzi et al. (2003a)[c]
	[1.5]	[0.01]	12–86	Strenn et al. (2004)[a]
	2.6–5.7	0.9–2.1	60–70	Carballa et al. (2004)[a]
	5.7	0.18	97 ± 4	Quintana et al. (2005)[b]
	28.0	3.0	98	Roberts and Thomas (2005)[a]
	2–3	0.6–0.8	53–79	Tauxe-Wuersch et al. (2005)[c]
	13.1 ± 4	0–3.8	78–100	Lindqvist et al. (2005)[c]
Ketoprofen	0.41–0.52	0.008–0.023	98	Thomas and Foster (2004)
	[0.55]	[0.18–0.3]	48–69	Stumpf et al. (1999)[b]
	5.7	n.r.		Metcalfe et al. (2003a)[a]
	0.47	0.18	62 ± 21	Quintana et al. (2005)[b]
	0.25–0.43	0.15–0.24	8–53	Tauxe-Wuersch et al. (2005)[c]
	2.0 ± 0.6	0–1.25	51–100	Lindqvist et al. (2005)[c]
Mefenamic acid	1.6–3.2	0.8–2.3	2–50	Tauxe-Wuersch et al. (2005)[c]
	0.20	0.34	0	Roberts and Thomas (2005)[a]
Naproxen			66	Ternes (1998)[b]
	40.7	12.5	40–100	Metcalfe et al. (2003a)
	10.3–12.8	n.d.-0.023	100	Thomas and Foster (2004)
	[0.6]	[0.1–0.54]	15–78	Stumpf et al. (1999)[b]
			93 (42–93)	Andreozzi et al. (2003a)[c]
	1.8–4.6	0.8–2.6	40–55	Carballa et al. (2004)[a]
	0.95	0.27	71 ± 18	Quintana et al. (2005)[b]
	4.9 ± 1.7	0.15–1.9	55–98	Lindqvist et al. (2005)[c]
Paracetamol	6.9	0	100	Roberts and Thomas (2005)[a]
β-Blocker				
Metoprolol	n.r.	n.r.	83	Ternes (1998)[b]
	n.r.	n.r.	10 (0–10)	Andreozzi et al. (2003a)[c]

Table 2 (Continued)

Compound	Influent concentration (μg/L)	Effluent concentration (μg/L)	Maximal removal (%)	Reference
Propranolol	n.r.	n.r.	96	Ternes (1998)[b]
	70	304	0	Roberts and Thomas (2005)[a]
Atenolol	n.r.	n.r.	<10 (0–10)	Andreozzi et al. (2003a)[c]
Blood lipid lowering agents				
Bezafibrate	[1.18]	[0.6–0.84]	27–50	Stumpf et al. (1999)[b]
	n.r.	n.r.	83	Ternes (1998)[b]
	[5]	[0.01]	10–97	Strenn et al. (2004)[a]
	0.6	0.2		Metcalfe et al. (2003a)[a]
	2.6	0.24	91 ± 4	Quintana et al. (2005)[b]
	0.42 ± 0.3	0–0.85	15–100	Lindqvist et al. (2005)[c]
Gemfibrozil	n.r.	n.r.	69	Ternes (1998)[b]
	[0.3]	[0.18–0.28]	16–46	Stumpf et al. (1999)[b]
	n.r.	n.r.	75 (10–75)	Andreozzi et al. (2003a)[c]
	0.7	1.3	n.r.	Metcalfe et al. (2003a)[a]
Fenofibric acid	[0.44]	[0.22–0.4]	6–45	Stumpf et al. (1999)[b]
	n.r.	n.r.	64	Ternes (1998)[b]
Clofibric acid	n.r.	n.r.	6–50	Stumpf et al. (1996)
	[1]	[0.68–0.88]	15–34	Stumpf et al. (1999)[b]
	n.r.	n.r.	51	Ternes (1998)[b]
	0.15–0.25	0.15–0.25	0	Tauxe-Wuersch et al. (2005)[c]
	0.34	0	91	Roberts and Thomas (2005)[a]
Neuroactive compounds				
Carbamazepine	n.r.	n.r.	7–8	Ternes (1998)[b]
	0.7	0.7	<50	Metcalfe et al. (2003a)[a]
	n.r.	n.r.	8	Heberer (2002)
	[1.5]	n.r.	4	Clara et al. (2004)[a]
	n.r.	[1.5]	53 (0–53)	Andreozzi et al. (2003a)[c]
Diazepam	0.59–1.18	0.1–0.66	93	Van Der Hoeven (2004)
Various				
Ethinylestradiol	0.003	0.0004	85	Baronti et al. (2000)
Clotrimazole	0.031	0.14	55	Roberts and Thomas (2005)[a]
Ifosfamide	0.007–0.029	0.010–0.043	0	Kümmerer et al. (1997)[a]
Tamoxifen	0.15	0.20	0	Roberts and Thomas (2005)[a]
X-ray contrast media	0.18–7.5	0.14–8.1	0	Ternes and Hirsch (2000)[b]

Data estimated from graphical data are in square brackets. n.r.: not reported.
[a] Median concentrations or percent.
[b] Average concentrations or percent.
[c] Maximal concentrations or percent.

other factors such as temperature and weather. For instance, diclofenac showed largely different elimination rates between 17% (Heberer 2002) and 69% (Daughton and Ternes 1999), and 100% (Hollert *et al.* 2000).

Once in surface waters, biotransformation through biodegradation occurs, but abiotic transformation reactions are probably more important. Whereas hydrolysis is generally negligible for environmentally relevant human drugs, photodegradation sometimes plays an important role at the water surface. Photolysis has been shown to be the main removal process for diclofenac in surface water (Buser *et al.* 1998b). For additional pharmaceuticals (sulphamethoxazole, ofloxacin and propranolol) laboratory experiments indicate direct and indirect photolysis as an important removal process (Andreozzi *et al.* 2003b). Carbamazepine

and clofibric acid, both compounds that are marginally processed in STP, have been shown to undergo slow photodegradation in salt- and organic free water with estimated half-lives in the range of 100 days at latitudes of 50°N in winter (Andreozzi *et al.* 2003b). The efficiency of photodegradation depends, besides substance properties, on the strength of the solar irradiation, and therefore on latitude and season, and on constituents present in the water that may act as photosensitizers generating hydroxyl radicals and singlet oxygen (i.e. nitrates, humic acids). Some adsorption to particles may occur. Laboratory batch studies to characterize the sorption behaviour of carbamazepine, diclofenac and ibuprofen in sandy sediments show that sorption coefficients were generally quite low (Scheytt *et al.* 2005). Diclofenac and ibuprofen are carboxylic acids with pKa values of 4.16 and 4.52 and these weak acids are negatively charged at pH of ambient water and sediment.

There are no information about the bioaccumulation potential of pharmaceuticals in biota or food webs with the exception of diclofenac, accumulating in the prey of vultures (Oaks *et al.* 2004), fluoxetine, sertraline and the SSRI metabolites norfluoxetine and desmethylsertraline detected in fish (Brooks *et al.* 2003). Diclofenac bioconcentration factors were 10-2'700 in the liver of fish and 5-1'000 in the kidney, depending on exposure concentrations (Schwaiger *et al.* 2004).

A few cases were reported, where pharmaceuticals were detected in drinking water (Heberer and Stan 1996) and groundwater (Daughton and Ternes 1999; Lilius *et al.* 1994). Ozonation, granulated activated carbon, and advanced oxidation have been shown as efficient removal processes. In drinking water, this has been shown for diclofenac, while clofibric acid and ibuprofen were oxidized in laboratory experiments mainly by ozone/H_2O_2 (Zwiener and Frimmel 2000). The elimination of selected compounds (bezafibrate, clofibric acid, carbamazepine, diclofenac) during drinking water treatment was investigated in laboratory experiments and waterworks (Daughton and Ternes 1999). No significant removal was observed in batch experiments with sand, indicating low sorption properties and persistence. Flocculation using iron(III) chloride was ineffective, but ozonation was in some cases very effective in eliminating these polar pharmaceuticals. However, clofibric acid was stable and not eliminated, even with filtration using granular activated carbon, which was effective for the other compounds. The removal of pharmaceuticals and other polar micropollutants can therefore only be assured using more advanced techniques such as ozonation, activated carbon or membrane filtration (Daughton and Ternes 1999). However, the economic consequences have to be evaluated carefully before investing into these advanced treatment technologies on a larger scale.

Environmental concentrations

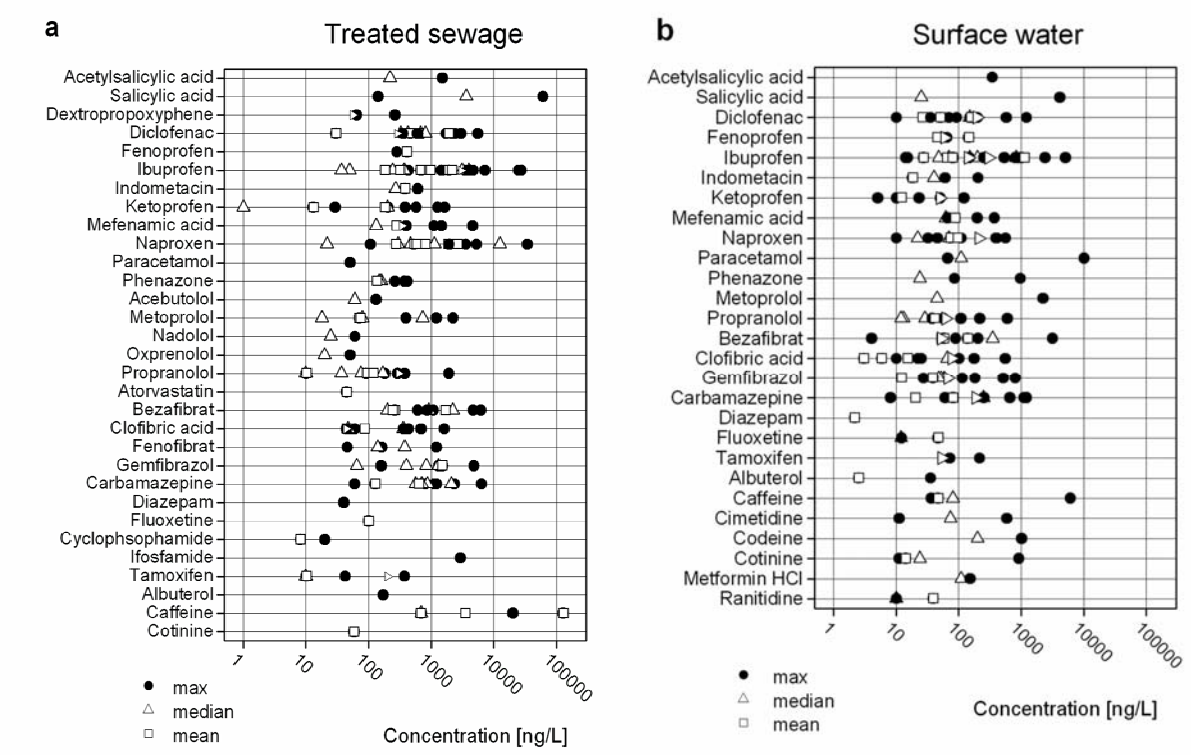
The occurrence of pharmaceuticals was first reported in the U.S.A. in treated wastewater, were clofibric acid in the range of 0.8-2 µg/L was found (Garrison *et al.* 1976). Subsequently, pharmaceuticals were detected in the U.K. in 1981 in rivers up to 1 µg/L (Richardson and Bowron 1985), and ibuprofen and naproxen were identified in wastewaters in Canada (Rogers *et al.* 1986). In the last few years, knowledge about the environmental occurrence of pharmaceuticals has increased to a large extent due to new analytical techniques able to determine polar compounds at trace quantities. This also holds for the steroid hormones contained in contraceptive pills such as 17□-ethinylestradiol (EE2), which is linked to biological effects in fish (Desbrow *et al.* 1998; Stumpf *et al.* 1996). Data on environmental concentrations up to 2004 have been compiled and reviewed (e.g. Daughton and Ternes 1999; e.g. Halling-Sorensen *et al.* 1998; Heberer 2002; Kümmerer 2004). Here, we give a summary on environmental concentrations focusing on most recent analytical data with the ultimate aim to relate them to ecotoxicological data. First, we give a general overview on the occurrence of pharmaceuticals in general and in different environmental media, and subsequently present data on the different pharmaceutical classes.

Recent studies reported concentrations of a wide range of about 80-100 pharmaceuticals from many classes of drugs (antiinflammatory, beta-blockers, sympathomimetics, antiepileptics, lipid regulators, antibiotics etc.) and some of their metabolites in many countries in treated sewage, rivers and creeks, seawater, groundwater and even drinking water. Ternes (1998) reported on the occurrence of 32 pharmaceuticals belonging to different medicinal classes in German municipal STP effluents, river and stream waters. 20 different drugs and 4 corresponding metabolites including antiinflammatory drugs (salicylic acid, diclofenac, ibuprofen, indometacine, naproxen, phenazone), lipid regulators (bezafibrate, gemfibrozil, clofibric acid, fenofibric acid), beta-blockers (metoprolol, propranolol) and carbamazepine were found to be ubiquitously present in streams and river water in the ng/L range. In an extended monitoring study concentrations of 95 micro-pollutants in water samples of 139 streams downstream of urban areas and livestock production across the U.S.A. were detected (Kolpin *et al.* 2002). In some sites as many as 38 of the targeted 95 compound were detected in a single water sample (average number of compounds in a sample was seven). Among the most frequently detected compounds were steroids (although some data had to be withdrawn subsequently), an insect repellant (N,N-diethyltoluamide), caffeine, triclosan (an antimicrobial compound), antibiotics, a fire retardant, 4-nonylphenol and some pharmaceuticals. Analysis of the distribution of different drugs in the river Elbe and its tributaries between the source and the city of Hamburg, Germany, showed the presence of many pharmaceuticals. The main substances found were diclofenac, ibuprofen, carbamazepine, various antibiotics and lipid regulators (Wiegel *et al.* 2004). A similar contamination pattern was found in Italy in the river Po and Lambro (Calamari *et al.* 2003)

where at all sampling sites atenolol, bezafibrate, furosemide, and antibiotics were found and ranitidine, clofibric acid, diazepam were often detected. Kolpin et al. (2004) collected water samples upstream and downstream of selected towns and cities in Iowa, U.S.A., during high-, normal- and low-flow conditions to determine the contribution of urban centres to concentrations of pharmaceuticals and other organic wastewater contaminants in streams under varying flow conditions. Prescription drugs were only frequently detected during low-flow conditions.

Environmental concentrations of pharmaceuticals were mainly reported in STP effluents and in surface water in many countries, often at locations near STPs (Ashton *et al.* 2004; Gross *et al.* 2004; Halling-Sorensen *et al.* 1998; Kolpin *et al.* 2002). The occurrence of selected pharmaceuticals was also reported in the Tyne estuary in the U.K. with concentrations ranging from 4 to 2370 ng/L (Roberts and Thomas 2005). Figure 1 gives a summary on the concentrations of most frequently assessed pharmaceuticals in wastewater and surface water reported so far. In STP effluents a number of different pharmaceuticals occur at concentrations generally in the ng/L to µg/L range. In rivers, lakes and seawaters, they are in the ng/L range (Ashton *et al.* 2004; Buser *et al.* 1998b; Hollert *et al.* 2000; Kolpin *et al.* 2002; Weigel *et al.* 2002). The rather persistent antiepileptic carbamazepine, and clofibric acid, a metabolite of the

Figure 1: Concentration of pharmaceuticals in treated wastewater (**a**) and surface water (**b**). References: (Andreozzi *et al.* 2003b; Calamari *et al.* 2003; Daughton and Ternes 1999; Gross *et al.* 2004; Halling-Sorensen *et al.* 1998; Hollert *et al.* 2000; Jones *et al.* 2002; Kolpin *et al.* 2002; Kümmerer 2004; Lindqvist *et al.* 2005; Metcalfe *et al.* 2003a; Metcalfe *et al.* 2003b; Oaks *et al.* 2004; Quintana *et al.* 2005; Roberts and Thomas 2005; Stackelberg *et al.* 2004; Stuer-Lauridsen *et al.* 2000; Tauxe-Wuersch *et al.* 2005; Weigel *et al.* 2004; Wiegel *et al.* 2004)



lipid lowering agents clofibrate, etofibrate and etofyllin clofibrate, have been detected with few exceptions in STP effluents, freshwater (rivers and lakes) and even in seawater (Buser *et al.* 1998b; Weigel *et al.* 2002). In surface water, carbamazepine is found with maximal concentrations of 1.2 µg/L (Wiegel *et al.* 2004) and clofibric acid at 0.55 µg/L (Daughton and Ternes 1999). Carbamacepin contamination is widespread. In 44 rivers across the U.S.A. average levels were 60 ng/L in water and 4.2 ng/mg in the sediment (Thaker 2005). Frequently, the analgesic ibuprofen and its metabolites were detected in STP effluents (Boyd *et al.* 2003; Buser *et al.* 1999; Daughton and Ternes 1999; Weigel *et al.* 2004), in surface water of up to 1 µg/L (Kolpin *et al.* 2002), and in seawater (Hollert *et al.* 2000; Weigel *et al.* 2004). In a monitoring study in the U.K. propranolol (median level 76 ng/L) was always found in STP effluents, whereas diclofenac (median 424 ng/L) was found in 86%, ibuprofen (median 3086 ng/L) in 84%, mefenamic acid (median 133 ng/L) in 81%, dextropropoxyphene (median 195 ng/L) in 74%, and trimethoprim (median 70 ng/L) in 65% of the samples (Ashton *et al.* 2004). In the corresponding receiving streams, fewer compounds and lower levels were found.

Some drinking waters (Heberer and Stan 1996; Putschew *et al.* 2000; Stackelberg *et al.* 2004; Stumpf *et al.* 1999; Zuccato *et al.* 2000), groundwaters (Daughton and Ternes 1999; Lilius *et al.* 1994), and landfill leachates (Lilius *et al.* 1994) contain pharmaceuticals in the ng/L range, in some cases up to µg/L. Phenazone, propiphenazone and clofibric acid were found in samples of potable water collected in the vicinity of Berlin, Germany (Heberer and Stan 1997; Reddersen *et al.* 2002). Several polar pharmaceuticals such as clofibric acid, carbamazepine, and X-ray contrast media can occur in groundwater. In the following, current knowledge about major pharmaceuticals of different therapeutic classes is summarized.

Analgesics and antiinflammatory drugs. The widely used non-steroidal antiinflammatory drugs (NSAID) ibuprofen, naproxen, diclofenac and some of their metabolites (e.g. hydroxyl-ibuprofen and carboxy-ibuprofen) are very often detected in sewage and surface water. Ternes (1998) reported levels in sewage exceeding 1 µg/L, and in effluents of conventional STP (mechanical clarification and biological treatment) concentrations often approach or exceed 0.1 µg/L in the U.S.A. (Gross *et al.* 2004). The deacylated, more active form of acetyl salicylic acid, salicylic acid, has been found in many municipal wastewaters at levels up to 4.1 µg/L (Daughton and Ternes 1999), 13 µg/L (Farré *et al.* 2001; Heberer 2002) or even 59.6 µg/L with median levels of 3.6 µg/L (Metcalfe *et al.* 2003a). However, salicylic acid may also derive from other sources. Similar to acetyl salicylic acid, acetaminophen (paracetamol) is well removed from STP. However, up to 10 µg/L (median 0.11 µg/L) acetaminophen has been found in 24% of samples from U.S. streams (Kolpin *et al.* 2002). The analgesic codeine was detected in 7% of samples at median concentrations of 0.01 µg/L.

In many countries diclofenac was frequently detected in wastewater in the µg/L range, and in surface water at lower levels (Buser *et al.* 1998b; Daughton and Ternes 1999; Farré *et al.* 2001; Heberer 2002; Heberer and Stan 1997; Sedlak and Pinkston 2001; Stumpf *et al.* 1999). This also holds for ibuprofen (Buser *et al.* 1999; Daughton and Ternes 1999; Heberer and Stan 1997; Stumpf *et al.* 1999). Sometimes, high levels of up to 85 µg/L (Farré *et al.* 2001), or 24.6 µg/L (median 4.0 µg/L) were detected in STP effluents (Metcalfe *et al.* 2003a). In Norway, ibuprofen and its metabolites occurred in all sewage samples, and in seawater at concentrations of 0.1-20 µg/L (sum of ibuprofen and metabolites) (Weigel *et al.* 2004). In U.K. estuaries maximal concentration of 0.93 µg/L (median 0.05 µg/L) occurred (Hollert *et al.* 2000). Ibuprofen is significantly removed during sewage treatment, and metabolites such as hydroxy-ibuprofen occur in STP effluents. Kolpin et al. (2002) found ibuprofen in 10% of stream water samples with maximal concentrations of 1 µg/L (median 0.2 µg/L). In two stormwater canals levels of ibuprofen were up to 674 ng/L and of naproxen up to 145 ng/L (Boyd *et al.* 2004). Naproxen was also found at much higher level in Canadian STP effluents with median levels of 12.5 µg/L and maximal levels of up to 33.9 µg/L (Metcalfe *et al.* 2003a). Moreover, several other analgesics have been detected in sewage and surface water, but also in ground water and drinking water samples.

Beta-blockers. Several beta-blockers were identified in wastewater (Daughton and Ternes 1999; Sedlak and Pinkston 2001). Propranolol, bisoprolol and metoprolol were found at highest levels (0.59 µg/L, 2.9 µg/L and 2.2 µg/L respectively in surface water), with lower levels of nadolol (in surface water) and betaxolol (0.028 µg/L in surface water) (Daughton and Ternes 1999). Propranolol, metoprolol and bisoprolol have also been found in surface water, and sotalol in groundwater (Sacher *et al.* 2001).

Blood lipid lowering agents. Clofibric acid, the active metabolite from a series of widely used blood lipid regulators (clofibrate, etofyllin clofibrate, etofibrate) belongs to the most frequently found and reported pharmaceutical in monitoring studies. It has been found in numerous wastewaters, surface waters, in seawater (Buser *et al.* 1998a; Daughton and Ternes 1999; Stumpf *et al.* 1996), and at rather high concentrations in groundwater (4 µg/L) (Heberer and Stan 1997) and drinking water (0.07-0.27 µg/L) (Heberer and Stan 1997; Stumpf *et al.* 1996). Bezafibrate occurred in maximal concentrations of up to 4.6 and 3.1 µg/L (median 2.2 and 0.35 µg/L, respectively) in wastewater and surface water, respectively (Daughton and Ternes 1999; Stumpf *et al.* 1996). In addition, gemfibrozil, clofibric acid and fenofibric acid (metabolite of fenofibrate) have also been detected in sewage up to the µg/L level and in surface water (Daughton and Ternes 1999; Farré *et al.* 2001; Heberer 2002; Stumpf *et al.* 1999). Gemfibrozil was detected in 4% of streams at maximal levels of 0.79 µg/L (Kolpin *et al.* 2002).

Neuroactive compounds (antiepileptics, antidepressants). Of this category, the antiepileptic carbamazepine was detected most frequently and in highest concentration in wastewater (up to 6.3 µg/L) (Daughton and Ternes 1999), and at lower levels in other media (Andreozzi *et al.* 2003b; Heberer *et al.* 2002; Metcalfe *et al.* 2003b; Wiegel *et al.* 2004). Carbamazepine was found in every Canadian STP effluent sample at concentration up to 2.3 µg/L (Metcalfe *et al.* 2003b). This compound was found to occur ubiquitously in the river Elbe and its tributaries, Germany (Wiegel *et al.* 2004), exceeding 1 µg/L in other German surface waters (Daughton and Ternes 1999; Heberer 2002) and occurred in groundwater (Daughton and Ternes 1999; Sacher *et al.* 2001; Seiler *et al.* 1999). In U.S. rivers average levels were 60 ng/L in water and 4.2 ng/mg in the sediment (Thaker 2005). Carbamacepine was also found at average levels of 20.9 ng/mg solids of STP. Diazepam was present in 8 of 20 STP in Germany at relatively low concentrations of up to 0.04 µg/L (Daughton and Ternes 1999) whereas in Belgium it was found at concentration up to 0.66 µg/L (van der Ven *et al.* 2004). The antidepressant fluoxetine was also detected in STP effluents samples in Canada (Metcalfe *et al.* 2003a), and in U.S. streams, median concentrations of 0.012 µg/L were estimated (Kolpin *et al.* 2002). Primidone, an antiepileptic drug, has also been detected in sewage up to 0.6 µg/L (Heberer 2002).

Antineoplastics and antitumor agents. Pharmaceuticals used in cancer chemotherapy occur primarily in hospital effluents and only at lower concentrations in municipal wastewater. Ifosfamide and cyclophosphamide occur in concentrations of up to 4.5 µg/L in hospital wastewaters (Steger-Hartmann *et al.* 1997), and at ng/L in municipal wastewater (Kümmerer 2004; Steger-Hartmann *et al.* 1997). The occurrence of the antiestrogen tamoxifen used in breast cancer therapy was reported in U.K. wastewater, where concentrations in STP effluents ranged between 146-369 ng/L (Roberts and Thomas 2005). Tamoxifen was not reduced in the STP, and even found in estuarine waters (Tye estuary) at concentrations ranging from 27-212 ng/L with a median level of 53 ng/L (Hollert *et al.* 2000; Roberts and Thomas 2005).

Various other compounds. Many additional pharmaceuticals have been detected in sewage and surface water (Daughton and Ternes 1999; Heberer 2002; Kolpin *et al.* 2002). Here only a few of them will be mentioned. The stimulant caffeine and the nicotine metabolite cotinine were generally present in STP effluents and surface waters contaminated by drugs (Metcalfe *et al.* 2003b). Caffeine was generally found in U.S. streams at maximal levels of 6.0 µg/L (median 0.1 µg/L) (Kolpin *et al.* 2002) and this compound can even serve as an anthropogenic marker in aquatic systems due to its ubiquity in surface water, seawater (Weigel *et al.* 2004), and also in groundwater (Figure 1). The antiacid cimetidine and ranitidine were estimated to occur in U.S. streams at concentrations of 0.58 µg/L and 0.01 µg/L, respectively, and they were detected at a frequency of 10 % and 1%, respectively (Kolpin *et al.* 2002). X-ray contrast media are very persistent. Iopamidol has been found in municipal wastewater as high as 15 µg/L, in surface

water (0.49 µg/L) and groundwater (Daughton and Ternes 1999; Putschew *et al.* 2000). Iopromide was detected at 2-4 µg/L in surface water, and up to 21 µg/L in STP (Putschew *et al.* 2000), but showed degradation in the laboratory (Steger-Hartmann *et al.* 2002). Hospital wastewater was also a source of gadolinium (Kümmerer 2004). The antidiabetic compound metformin was observed in 5% of stream water samples with estimated levels of 0.11 µg/L (Kolpin *et al.* 2002). Bronchodilators (β_2-sympathomimetics terbutalin and salbutamol) were also detected in sewage in a few cases not exceeding 0.2 µg/L (Daughton and Ternes 1999).

Steroidal hormones have been reported on in many reports, and in our review we only summarize knowledge about the synthetic estrogen EE2 and mestranol contained in contraceptive pills. These steroids have been found in numerous studies in many countries in Europe, Canada, the U.S.A., Japan, Brazil etc. both in wastewater and surface water. A survey in the U.S.A. showed that maximal and median EE2 concentrations were as high as 831 and 73 ng/L, respectively, and levels of mestranol were 407 and 74 ng/L, respectively (Kolpin *et al.* 2002). They were detectable in 16 % and 10 % of the streams sampled. Generally, median concentrations are much lower being in the range of non detectable up to 9 ng/L in treated wastewater in several countries (Baronti *et al.* 2000). Typical wastewater effluent concentrations are 0.5 ng/L and they are even lower in surface water. However, these concentrations must put into the perspective of their high biological activity accounting for potential estrogenic effects in fish.

Exposure and fate models are increasingly being used to estimate environmental concentrations without analytical chemical measurements. Some exposure models have been developed for drugs (e.g. PhATE), others have been extended from general chemicals to pharmaceuticals (e.g. EPIWIN, GREAT-ER). These tools have been developed both for estimation of predicted environmental concentrations (PEC) and the behaviour of pharmaceuticals in the environment. A pharmaceutical assessment and transport evaluation model (PhATE) was developed to estimate concentrations of active pharmaceutical ingredients in U.S. surface waters (Sanderson *et al.* 2004). The PhATE model uses some for most hydrologic regions of the U.S. representative watersheds. For European surface waters an exposure simulation was developed for pharmaceuticals with the GREAT-ER (Geo-referenced Regional Exposure Assessment Tool for European Rivers) model, a tool developed for use within ecological risk assessment (ERA) schemes and river basin management (Schowanek and Webb 2002). The GREAT-ER software calculates the distribution of PEC's of consumer chemicals in surface waters, for individual stretches, as well as representative average PEC's for entire catchments. The system uses an ARC/INFO-ArcView (®ESRI) based Geographical Information System (GIS) for data storage and visualization, combined with simple mathematical models for prediction of the fate of chemicals.

For some estimates, measured environmental concentrations (MEC) are in agreement with the estimated PEC's, however, often, they are not as large differences occur between the models and the real world situation. The main reason is that different assumptions are made, which not always correspond to the real conditions in the environment. Consumption figures, metabolism in the organism, removal during sewage treatment plants and fate in the environment contain all uncertainties that may result in inappropriate estimates of PEC's. Moreover, detailed situations at a given site is not reflected by models integrating large geographical areas. Poor prediction performance of current models for many pharmaceuticals is one of the outstanding scientific issues with regard to the question of pharmaceuticals in the environment. It is hoped that the models are improving by further refining the mentioned uncertainties and may be developing to a useful and readily applicable regulatory tool (Sanderson *et al.* 2004).

Modes of actions in humans and mammals and occurrence of target biomolecules in lower vertebrates and invertebrates

Here, we briefly summarize the modes of actions of pharmaceutical classes and ask, whether or not similar target receptors and biomolecules exist in lower vertebrates and invertebrates. Knowledge about similar targets exists primarily for fish. In general, very little is known about possible counterparts of human target biomolecules of pharmaceuticals in invertebrates. In addition, some of the side effects in humans are discussed, giving hints to possible adverse effects in lower animals.

Analgesics and non-steroidal antiinflammatory drugs (NSAID). Non-steroidal antiinflammatory drugs act by inhibiting either reversibly or irreversibly one or both of the two isoforms of the cyclooxygenase enzyme (COX-1 and COX-2), which catalyze the synthesis of different prostaglandins from arachidonic acid (Vane and Botting 1998). Classical NSAID inhibit both COX-1 and COX-2 at different degrees, whereas new NSAID act more selectively on COX-2, the inducible form responsible for the inflammatory reactions. Differences in binding site size are responsible for the selectivity of these drugs (Gierse *et al.* 1999; Kurumbail *et al.* 1997; Penning *et al.* 1997). NSAIDS are commonly used to treat inflammation and pain and to relieve fever, and sometimes they are also used for long-term treatment of rheumatic diseases.

Prostaglandins play a variety of physiological roles according to their cells source and target molecules. They are known to be involved in process such as inflammation and pain, regulation of blood flow in kidney, coagulation processes and synthesis of protective gastric mucosa (Mutschler 1996; Smith 1971; Vane 1971). Since NSAID inhibit non-specifically prostaglandin synthesis, most side effects, at least after long-term treatment, are related to the physiological

function of prostaglandins. In the kidney, prostaglandins are involved in maintenance of the equilibrium between vasoconstriction and vasodilatation of the blood vessel that supply glomerular filtration. Renal damages and renal failure after chronic NSAID treatment seems to be triggered by the lack of prostaglandins in vasodilatation-induction. Gastric damages are thought to be caused by inhibition of both COX isoforms (Wallace 1997; Wallace *et al.* 2000). In contrast, liver damages are apparently due to building of reactive metabolites (e.g. acyl glucuronides) rather than inhibition of prostaglandins synthesis (Bjorkman 1998).

The mode of action of paracetamol is not yet fully elucidated. It seems that this drugs acts mainly by inhibiting the cyclooxygenase of the central nervous system and it does not have antiinflammatory effects, because of the lack of inhibition of peripheral cyclooxygenase involved in inflammatory processes. Adverse effects of paracetamol are mainly due to formation of hepatotoxic metabolites, primarily N-acetyl-p-benzoquinone imine, synthesized when the availability of glutathione is diminished in liver cells. Acetaminophen widely used in many analgesic/antipyretic medications induces proliferation of cultured breast cancer cells via estrogen receptors without binding to them, but has no estrogenic activity in rodents (Harnagea-Theophilus *et al.* 1999). The consequences of these observations are not clear.

In fish an inducible COX-2 homologue has been found to be expressed in macrophages in rainbow trout (*Oncorhynchus mykiss*) and the translation product of the COX gene was found to have a high homology of 83-84% and 77% to its human counterpart COX-2 and COX-1, respectively (Zou *et al.* 1999). Also in goldfish, macrophages express a COX enzyme, which is an equivalent to mammalian COX-2 (Zou *et al.* 1999). A COX-1 and COX-2 homologue was cloned from brook trout ovary (Roberts *et al.* 2000), and recently, a shark COX was cloned in dogfish *Squalus acanthias* having 68% and 64% homology to mammalian COX-1 and COX-2, respectively (Chong *et al.* 2000). Prostaglandins are formed in a diverse range of vertebrates and invertebrates. However, in lower invertebrates such as corals, their synthesis is independent of COX, involving other enzymes (Song and Brash 1991). In arthropods and molluscs, COX-like activity is apparently responsible for the formation of prostaglandins, but these enzymes have not been purified and characterized (Pedibhotla *et al.* 1995). In birds, prostaglandins play a role in the biosynthesis of egg shells and treatment with the COX-inhibitor indometacine resulted in egg shell thinning (Lundholm 1997).

Beta-blockers. Beta-blocker act by competitive inhibiting beta-adrenergic receptors and they are used in the treatment of high blood pressure (hypertension), and to treat patients after heart attack to prevent further attacks. The adrenergic system is involved in many physiological functions such as regulation of the heart oxygen need and beating, vasodilatation mechanisms of blood vessels, and bronchodilation. Furthermore, the adrenergic system is also known to

interact with carbohydrate and lipid metabolisms, mainly in response to stress needs such as starvation (Jacob *et al.* 1998).

β-Adrenoceptors are 7-transmembrane receptor proteins coupled with different G-proteins that ultimately enhance the synthesis of the second messenger signaling molecules cAMP (Rang *et al.* 2003). According to medical needs beta-blockers may selectively inhibit one or more β-receptors types; for example β_2-blockers are used to treat hypertension avoiding cardiac effects, since this receptor subtype is not found in the heart. Selectivity is based on difference in chemical groups added to compounds that are able to enhance the interactions with amino acids of the transmembrane domains. Some of the beta-blockers (e.g. propranolol, a beta1-adrencoceptor antagonist) have the ability to cause cell membrane stabilization, whereas other (e.g. metoprolol) have no membrane stabilizing activity (Doggrell 1990). Side effects of this therapeutic class are mainly bronchoconstriction and disturbed peripheral circulations (Hoffman and Lefkowitz 1998; Scholze 1999). Due to their lipophility they are supposed to pass the blood brain barrier and to act in the central nervous system (Soyka 1984, 1985).

β-Adrenoceptors where found in fish (*Oncorhynchus mykiss*) liver, red and white muscle with a high degree of sequence conservation with other vertebrate homologues. They are also supposed to play similar role as in humans (Nickerson *et al.* 2001). The presence of a β_2-adrenoceptor subtype was also suggested by binding studies to occur in liver membranes of other fish and amphibians. β_2-Adrenoceptors of rainbow trout (Nickerson *et al.* 2001) show a high degree of amino-acid sequence conservation with other vertebrate β_2-adrenoceptors. Frog-(Devic *et al.* 1997) and turkey β_1-adrenoceptors (Yardeny *et al.* 1986) are similar to mammalian β_1-adrenoceptors. In rainbow trout, the β_2-adrenoceptor gene is highly expressed in the liver, red and white muscle, with lower expression in gills, heart, kidney and spleen (Nickerson *et al.* 2001). Clenbuterol or ractopamine that function in mammals as β-agonist were found in rainbow trout to show a somewhat different reaction. Clenbuterol displayed only partial agonist activities and the small effects of ractopamine may be related to low affinity for the trout β_2-adrenoceptor. Agonist regulation of the trout hepatic β_2-adrenoceptors may involve down-regulation of the receptors without affecting responsiveness (Dugan *et al.* 2003). Differences in the structure and function of the receptors may be responsible for differences in the affinity with β-blockers and mechanisms triggered by these drugs.

Whereas mammals have 3 $\alpha_2$adrenoceptors, five distinct α_2-adrenoceptor genes have been found expressed in zebrafish (Ruuskanen *et al.* 2005). Localization of the α-adrenoceptos in zebrafish shows marked conservation when compared with mammals. The α_2-adrenergic system is functional in zebrafish as demonstrated by marked locomotor inhibition and lightening of skin color induced by the specific α_2-adrenoceptor agonist dexmedetomidine, similar to mammals. Both effects were antagonized by the specific α_2-adreneceotor antagonist

atipamezole. The α-adrenoceptor agonists medetomidine and clonidine are being investigated as potential antifouling agents preventing the settlement of barnacles on ship halls (Dahlstrom *et al.* 2004). Settlement of larvae is inhibited at low concentrations of 0.25-2.5 µg/L. Additional pharmacological and biochemical investigations on α- and β-adrenoceptors of fish and other lower organisms are needed.

Blood lipid lowering agents. There are basically two types of anti-lipidemic drugs, namely statins and fibrates, the latter have been targeted analytically more often in the aquatic environment than the former. Both types are used to decrease the concentration of cholesterol (statins and fibrates) and triglycerides (fibrates) in the blood plasma. Statins as inhibitors of cholesterol synthesis act by inhibiting the 3-hydroxymethylglutaril coenzym A reductase (HMG-CoA), responsible for the limiting step in the cholesterol synthesis, namely the conversion of HMG-CoA to mevalonate (Laufs and Liao 1998). As a consequence of the intracellular cholesterol depletion, the expression of LDL receptors in hepatocyte membranes is increased and therefore, the resorption of LDL-cholesterol from blood plasma. Due to interactions of statins with mevalonate metabolism, multiple additional effects occur (antiinflammatory, antioxidative). There is also evidence that statins affect juvenile hormone synthesis in insects (Debernard *et al.* 1994), as fluvastatin completely suppressed its biosynthesis *in vitro*, and in the mandibular organo of lobsters (Li *et al.* 2003).

In contrast, effects of fibrates are mediated, at least in part, through alterations in transcription of genes encoding for proteins controlling lipoprotein metabolism. Fibrates act probably by activating the lipoprotein lipase enzyme, which is mainly responsible for the conversion of very low density lipoprotein (VLDL) to high density lipoproteins (HDL), decreasing therefore plasma triglycerides concentration (Staels *et al.* 1998). Binding of fibrates to peroxisome proliferator-activated receptors (PPARs), nuclear receptors known to be activated during different cellular pathways, stimulates the expression of several lipid regulatory proteins such as, for example, the lipoprotein lipase (Staels *et al.* 1998). To date, three subtypes of PPAR have been described; PPARα is involved in peroxisome proliferation and plays a pivotal role in controlling hepatic lipid metabolism (Schoonjans *et al.* 1996), whereas PPARβ has diverse roles in basic lipid metabolism, and PPARγ plays a key role in the differentiation of adipocytes (Kersten *et al.* 2000). Heterodimerization of PPAR's with the retinoid X receptor and their binding to response elements in the promoter regions of genes leads to their activation.

Fibrates stimulate cellular fatty acid uptake, conversion to acetyl-CoA derivatives, and catabolism by the beta-oxidation pathways, which, combined with a reduction in fatty acid and triglyceride synthesis, results in a decrease in VLDL production (Staels *et al.* 1998). Hepatic damages may occur after chronic exposure to fibrates in rat (Castano *et al.* 1994) (and this is thought to be related to inhibition of mitochondrial oxidative phosphorylation (Keller *et al.* 1992).

Furthermore, fibrates caused in rodents a massive proliferation of peroxisomes (Hess *et al.* 1965). Strong correlation between fibrates exposure and hepatocarcinogenicity in rodents were found, while this was not observed in humans (Cajaraville *et al.* 2003). These findings increase the interest for ecotoxicological impact of this therapeutic class of drugs.

PPAR genes have been found in fish such as plaice (Leaver *et al.* 1998) and Atlantic salmon (Ruyter *et al.* 1997) and zebrafish (Ibabe *et al.* 2002). Fish PPARs display an amino acid sequence identity of 43-48 % to the human and amphibian PPARγ (Andersen *et al.* 2000). All PPAR forms have been found in zebrafish, and PPARα was mainly expressed in hepatocyte and tissues that catabolize high amounts of fatty acids (Ibabe *et al.* 2002). Furthermore, PPARγ was shown to be induced in response to clofibrate and benzafibrate in salmon hepatocytes (Ruyter *et al.* 1997), although their PPARγ seem to be less responsive than PPARγ of rodents (Andersen *et al.* 2000). All three PPAR receptors were found to already been expressed in the larval stage, with a similar tissue distribution pattern to that found in adult zebrafish (Ibabe *et al.* 2005a). Activators of PPARα include a variety of endogenously present fatty acids, leukotrienes and hydroxyeicosatetraenoic acids and drugs, such as fibrates (Cajaraville *et al.* 2003). PPARβ activators include fatty acids, prostaglandin A_2 and prostacyclin. PPARγ is the most selective receptor and prostaglandin J_2 has been described to be a specific ligand (Ibabe *et al.* 2005b). In isolated zebrafish hepatocytes, mRNA of both PPARα and PPARγ was induced by clofibrate at 0.5-2 mM, although to a low extent (Ibabe *et al.* 2005b). The physiological and toxicological roles of PPAR's have yet to be investigated, and their involvement in potential effects of lipid lowering drugs is not yet known. With regard to invertebrates, no information is currently available on the existence of PPARs, although extensive searches for nuclear receptors in cnidarians and platyhelminthes have been performed (Escriva *et al.* 1997).

Neuroactive compounds (antiepileptics, antidepressants). Among the many drugs interacting with the central nerve system (CNS), only a few will be considered as with respect to its occurrence in the aquatic environment. Antiepileptic drugs act on the CNS by decreasing the overall neuronal activity. This can be achieved either by blocking voltage-dependent sodium channels of excitatory neurons (e.g. carbamazepine), or by enhancing of inhibitory effects of the GABA neurotransmitter by binding on a specific site in the gamma subunit of the corresponding receptor (e.g. diazepam, member of benzodiazepine family) (MacDonald and Olsen 1994; Rogers *et al.* 1994; Study and Barker 1981). Evidence of the occurrence of the GABA system in fish (*Oncorhynchus mykiss*, (Cole *et al.* 1984; Meissl and Ekstrom 1991)) was found, whereas no studies have been found indicating the occurrence of sodium voltage dependent channels in fish or lower invertebrate.

Fluoxetine is a widely used antidepressant, which acts by inhibiting the re-uptake of serotonin. This neurotransmitter is involved in many mechanisms, namely hormonal and neuronal, and it is

also important in functions such as food intake and sexual behavior. A pump directs serotonin from the synapse space back to the presynapse, and serotonin re-uptake inhibitors (SSRI) inhibit this pump, thus increasing the serotonin level in the synapse space. Serotonin as a neurotransmitter occurs in lower vertebrates and invertebrates (Chong *et al.* 2000), however, the effects associated with this transmitter are different, and so are possibly the effects of SSRI. Serotonin mediates, among others, endocrine functions in aquatic organisms such as fingernail claims (*Sphaerium striatinum*, (Chong *et al.* 2000)) and Japanese medaka (*Oryzias latipes*, (Brooks *et al.* 2003; Chong *et al.* 2000)). Fluoxetine and sertraline and the SSRI metabolites norfluoxetine and desmethylsertraline have been detected in fish sampled from wild in the U.S., and therefore reflect a bioaccumulation potential (Brooks *et al.* 2003). Whether the accumulated levels of 1.6 ng/g fluoxenine and 4.3 ng/g sertraline found in brain have effects on the nervous system of fish has yet to be investigated.

Cytostatics compounds and cancer therapeutics. Another potential interesting class of compound is represented by cytostatic pharmaceuticals interacting with cell proliferation. There are different modes of actions of the different compounds. For example methotrexate acts as a potent inhibitor of the folate dehydroreductase enzyme, which is responsible for the purine and pyrimidine synthesis (Rang *et al.* 2003; Schalhorn 1995). Doxorubicin is an intercalating substance inducing DNA-strand brakes (in humans, heart arrhythmia may be a side effect). Tamoxifen as an anti-estrogenic drug is used for breast cancer treatment and acts by competitive inhibiting the estrogenic receptor at least in mammary gland (Rang *et al.* 2003).

Various compounds. Cimetidine and ranitidine are compounds, which act by inhibiting the histamine receptors type 2 in the gastric system, thus inhibiting the acid secretion (antacid). These drugs are used to treat gastric ulceration. Since H_2-histamine receptors are found also in the brain, both drugs may elicit central nervous system reactions and side effects (Cannon *et al.* 2004). Peitsaro (2000) demonstrated the presence of H_3-histamin receptors in central nervous system of zebrafish (*Danio rerio*), but the lack of histamine in the periphery of this fish was also reported. However, interspecies differences may occur; cod and carp seem to have histamine and H_2-receptors in the periphery (Peitsaro *et al.* 2000).

Metformin is an antidiabetic agent, which mechanisms of actions are not well understood. It seems that this drugs acts by increasing the cellular use of glucose and inhibiting the gluconeogenesis. Metformin seems to act on insulin receptor by direct stimulation of the insulin receptor or indirectly through inhibition of tyrosine phosphatase (Holland *et al.* 2004).

Ecotoxicological effects

Pharmaceuticals are designed to target specific metabolic and molecular pathways in humans and animals, but they often have important side effects too. When introduced into the environment they may affect the same pathways in animals having identical or similar target organs, tissues, cells or biomolecules. As shown above, certain receptors in lower animals resemble those in humans, others however, are different or lacking, which means that dissimilar modes of actions may occur in lower animals. It is important in this respect to recognize that for many drugs, their specific modes of actions are not well known and often not only one, but many different modes of actions occur. Among other reasons, this makes specific toxicity analysis in lower animals difficult to perform. Despite this, toxicity experiments should be targeted and designed for specific targets of the pharmaceutical even in lower vertebrates and invertebrates, based on the hypothesis of similarity of modes of actions. However, current toxicity testing is not designed in this way, rather general and established test systems and traditional organisms according to guidelines are being used and traditional end points such as mortality are assessed.

Thus far, ecotoxicity testing merely provided indications of acute effects *in vivo* in organisms of different trophic levels after short-term exposure, and only rarely after long-term (chronic) exposures. These data are ultimately used for ecological risk assessments. Because of animal welfare and screening purposes, *in vitro* analyses are becoming more important, but they are not sufficient for assessing the toxicological profiles of a compound, particularly as a basis for risk analysis (Fent 2001). Beyond laboratory investigations, some mathematical models were developed to estimate or predict ecotoxicological effects. The most often applied quantitative structure activity relationship (QSAR) program is ECOSAR (online http://www.epa.gov/oppt/newchems/sarman.pdf) (Sanderson *et al.* 2004). Despite serious drawbacks such as an inadequate structure coverage for pharmaceuticals, the program has been repeatedly applied to estimate pharmaceutical baseline toxicities (Cleuvers 2005; Jones *et al.* 2002; Sanderson *et al.* 2004). Both methods are helpful in estimating potential toxicity or the behavior of a compound in the environment, but they cannot replace *in vivo* or *in vitro* assays.

The current literature about ecotoxicological effects of human pharmaceutical deals mainly with the acute toxicity in standardized tests and it is generally focused on aquatic organisms. The influence of environmental parameters such as pH on toxicity has only rarely, or not yet been investigated. Such studies would be of importance for instance for acidic pharmaceuticals that may induce different toxicities depending on speciation at different ambient pH. Moreover, effects of drug metabolites have rarely been investigated. Phototransformation products of naproxen, for instance, showed higher toxicities than the parent compound, while genotoxicity was not found (Isidori *et al.* 2005). At contaminated sites, aquatic life is exposed over the entire

life cycle to these compounds. Chronic effects are less investigated and often even related to relative short-term exposures. However, long-term exposures are needed for an accurate environmental risk assessment. Here we summarize the current ecotoxicological data, focusing on specific modes of action of different therapeutic classes of pharmaceuticals, and covering many differences in methods, species and time of exposure. These data are then related to environmental levels in order to assess the potential hazard for the different classes of pharmaceuticals and identify current research and knowledge gaps.

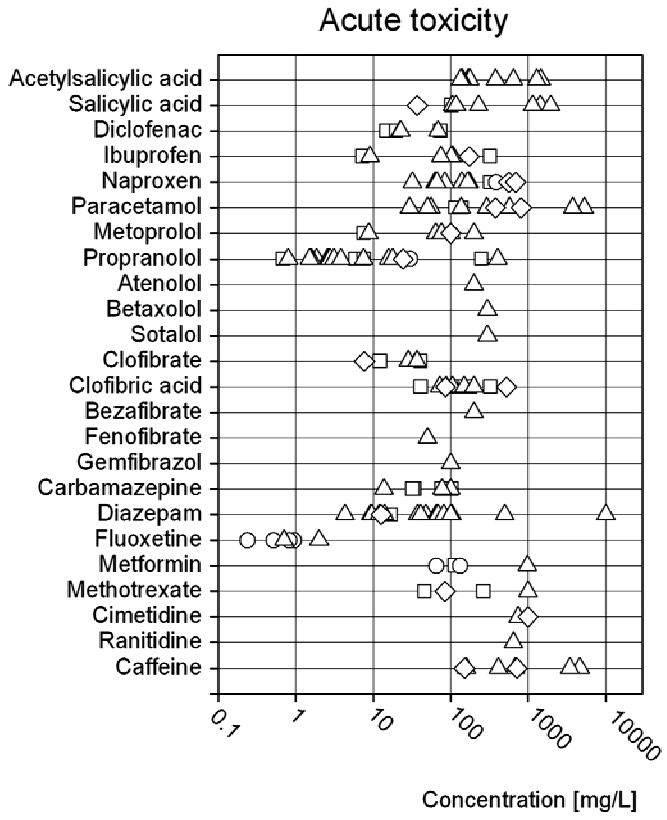
Acute effects

Pharmaceuticals are assessed for their acute toxicity by traditional standard tests according to established guidelines (e.g. OECD, U.S. EPA, ISO) using established laboratory organisms such as algae, zooplankton and other invertebrates and fish. Acute toxicity data of pharmaceuticals were compiled by Hallig-Sorensen et al. (1998) and Webb (2001), whereby in the latter, a list of about 100 human pharmaceuticals from different sources is given. By comparing different trophic levels, Webb (2001) suggested that algae were more sensitive to the listed pharmaceuticals than *Daphnia magna*, and fish were even less sensitive. However, such generalizations do not focus enough on the different modes of actions of a given pharmaceutical, and hence, differences in toxicity in different phyla. In the attempt to compare the different classes of pharmaceuticals in terms of acute toxicity, Webb (2001) noted that the most toxic classes were antidepressants, antibacterials and antipsychotics, but the range of responses within each of these categories was large, typically several orders of magnitude. In our present review, we provide and summarize additional and new data and discuss its ecotoxicological relevance covering different classes of human pharmaceuticals. The data originate from different sources, and studies were performed under different quality criteria (i.e. nominal versus measured exposure concentrations), making comparisons difficult.

Analgesics and non-steroidal antiinflammatory drugs (NSAID). In general, toxicity data vary for each pharmaceutical, however, diclofenac seems to be the compound having highest acute toxicity within the class of NSAID, since for all the tests performed the effect concentrations were below 100 mg/L (Figure 2). Short-term acute toxicity was analyzed in algae and invertebrates (Cleuvers 2003; Webb 2001), phytoplankton was found to react more sensitive [lowest EC_{50} (96 h) = 14.5 mg/L, (Ferrari *et al.* 2004)] than zooplankton [lowest EC_{50} (96h) = 22.43 mg/L, (Ferrari *et al.* 2004)]. There is no correlation between the acute toxicity in Daphnia and the lipophilicity as represented by log Kow (Figure 3). In general, not much is known about the acute toxicity to fish.

Beta-blockers. As shown in Figure 2, the acute toxicity of beta-blockers is not extensively studied, with the exception of propranolol. This compound shows the highest acute toxicity and highest log Kow as compared to other beta-blockers (Figure 3). This and the fact that it is a

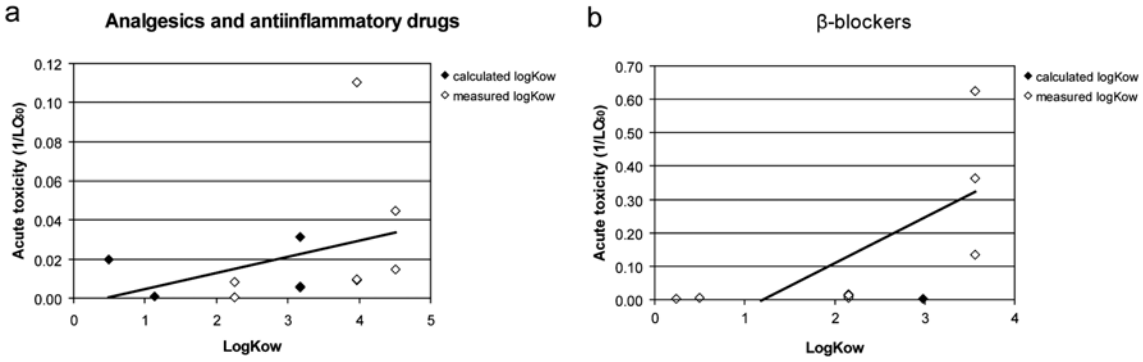
Figure 2: Acute toxicity of 24 different pharmaceuticals, belonging to different therapeutic classes to aquatic organisms. EC_{50} and LC_{50} for different organisms and different endpoint and exposure time are summarized. See text for details. *References*: (Brooks *et al.* 2003; Calleja *et al.* 1993, 1994; Chong *et al.* 2000; Cleuvers 2003, 2004; Ferrari *et al.* 2004; Halling-Sorensen *et al.* 1998; Henry *et al.* 2004; Henschel *et al.* 1997; Hernando *et al.* 2004; Hutchinson *et al.* 2003; Isidori *et al.* 2005; Kümmerer 2004; Lilius *et al.* 1994; Marques *et al.* 2004a, 2004b; Nunes *et al.* 2004; Villegas-Navarro *et al.* 2003; Webb 2001)



strong membrane stabilizer, whereas other investigated beta-blockers are not, may in part explain its higher toxicity (Doggrell 1990; Hutchinson *et al.* 2003). Comparison of toxicity is difficult in this case, since other beta-blockers, except metoprolol, were only analyzed in *D. magna* (Hernando *et al.* 2004). Metoprolol and verapamil caused the acceleration of the heart beat rate at low concentration, but lowered it at high concentrations in *D. magna* (Villegas-Navarro *et al.* 2003). For propranolol it seems that phyto- and zooplankton are more sensitive than fish. *Ceriodaphnia dubia* [EC_{50} (48 h) = 0.8 mg/L, (Ferrari *et al.* 2004)] displayed higher sensitivity than *D. magna* [EC_{50} (48 h) = 1.6 mg/L, (Hutchinson *et al.* 2003)] or other zooplankton organisms. Within phytoplankton, the microorganism *Synechococcus leopolensis* reacted most sensitive [EC_{50} (96 h) = 0.668 mg/L, (Ferrari *et al.* 2004)].

Blood lipid lowering agents. Similar to beta-blockers, acute toxicity of lipid lowering agents is not extensively reported. Clofibrate showed LC_{50} values in the range of 7.7 to 39.7 mg/L and can be classified as harmful to aquatic organisms. The fish *Gambusia holbrooki* [LC_{50} (96 h) = 7.7 mg/L, (Nunes *et al.* 2004)] seems the most sensitive organism to acute clofibrate

Figure 3: Relation between acute toxicity (LC_{50}) of analgesics and antiinflammatory drugs (y=0.0082x-0.0034; R^2=0.1202; ANOVA not significant) (**a**) and β-blockers (y=0.1386x-0.1709; R^2=0.4301; ANOVA significant; p<0.02) (**b**) and octanol-water partition coefficients of the compounds ($LogK_{ow}$); calculated and measured values are given in different symbols. Acute toxicity of *Daphnia magna* refers to immobilisation after 48 hours (LC_{50} value). *References*: Acute toxicity*:* (Brooks *et al.* 2003; Calleja *et al.* 1993; Cleuvers 2003, 2004; Ferrari *et al.* 2004; Halling-Sorensen *et al.* 1998; Henschel *et al.* 1997; Hernando *et al.* 2004; Hutchinson *et al.* 2003; Lilius *et al.* 1994; Marques *et al.* 2004a, 2004b; Villegas-Navarro *et al.* 2003). $LogK_{ow}$, in between parenteses: acetylsalicylic acid (1.13) (Sanderson *et al.* 2004); salicylic acid (2.26) (Hansch *et al.* 1995); diclofenac (4.51), ibuprofen (3.97) (Avdeef *et al.* 1998); naproxen (3.18) (Cleuvers 2004); paracetamol (0.49) (Henschel *et al.* 1997); atenolol (0.5) (Griffin *et al.* 1999); betaxolol (2.98) (Sanderson *et al.* 2004); metoprolol (2.15), propranolol (3.56) (Hardman *et al.* 1996); sotalol (0.24) (Hansch *et al.* 1995).



concentrations studied so far. The known rodent peroxisome proliferator gemfibrozil injected to rainbow trout led to significant increases in fatty acyl-CoA oxidase (FOA) activity at doses of 46-152 mg/kg/day (Scarano *et al.* 1994). Significant dose-related increases in peroxisomal FOA were observed after exposure of rainbow trout primary hepatocytes to clofibric acid, and ciprofibrate, but not with gemfibrocil (Donohue M. *et al.* 1993). The *in vitro* activity in these fish is weak.

Neuroactive compounds (antiepileptics, antidepressants). The serotonin re-uptake inhibitor fluoxetine is apparently the most acute toxic human pharmaceutical reported so far with acute toxicity ranging from EC_{50} (48 h, alga) = 0.024 mg/L (Brooks *et al.* 2003) to LC_{50} (48 h) = 2 mg/L (Kümmerer 2004). For benthic organisms, acute toxicity is in the range of 15-43 mg/kg sediment [*Chironomus tentans* LC_{50} (10 d) = 15.2 mg/kg, *Hyalella azteca* LC_{50} (10 d) = 43 mg/kg, (Brooks *et al.* 2003)]. Fluoxetine seems to stronger affect phytoplankton than other aquatic organisms.

Diazepam and carbamazepine, both antiepileptics, can be classified as potentially harmful to aquatic organisms, because most of the acute toxicity data are below 100 mg/L. For both compounds it seems that *D. magna* is affected more than other species, but the reasons for the higher susceptibility is not known. Acute toxicity of carbamazepine was found at 17.2 mg/L in *Daphnia* and at 34.4 mg/L in midges, but growth was inhibited at 12.7 mg/L in *Daphnia* and at 9.2 mg/L in midges (Thaker 2005).

Cytostatic compounds and cancer therapeutics. Acute toxicity of methotrexate on highly proliferative species, namely the ciliate *Tetrahymena pyriformis,* indicated acute effects [EC_{50} (48 h) = 45 mg/L*,* (Henschel *et al.* 1997)]. Teratogenicity in fish embryos was observed at even higher concentrations [EC_{50} (48 h) = 85 mg/L, (Henschel *et al.* 1997)].

The acute toxicity data summarized in Figure 2 shows that 17% of the pharmaceuticals displayed an acute toxicity below 100 mg/L, and for fluoxetine, all toxicity values were below 10 mg/L. On the other hand, 38 % of the pharmaceuticals such as acetylsalicylic acid, betaxolol, sotalol, bezafibrate, gemfibrozil, bezafibrate, cimetidine and ranitidine displayed LC_{50} values higher than 100 mg/L, which, according to EU-Directive 93/67/EEC (Commission of the European Communities, 1996), are classified as not being harmful for aquatic organisms. The other pharmaceuticals (45%) displayed a considerable variability of acute toxicity values, spreading over a wide range, thus making a classification difficult.

Variability of data both within the same and between different species is obvious. Different actual exposure concentrations (only nominal concentrations were used in the determination of the endpoints), different sensitivities of used clones, different laboratory performances are among the reasons for variability within the same species (for example, clofibric acid toxicity in *D. magna* varies between 72 and 200 mg/L; the LC_{50} (48h) of acetyl salicylic acid varies between168 mg/L (Calleja *et al.* 1994) and 1468 mg/L (Lilius *et al.* 1994); the LC_{50} (24h) of diazepam varies between 9.6 mg/L (Calleja *et al.* 1993) and 10‘000 mg/L (Calleja *et al.* 1994). Depending on the quantity and quality of data, ranges of acute toxicity values span one to two orders of magnitude, in some cases such as propranolol or diazepam, the species differences are quite large, spanning 3-4 orders of magnitude. When different categories are compared, a tendency of lower LC_{50} (EC_{50}) values is found for beta-blockers and neuroactive drugs as compared to antiinflammatory drugs or various other compounds.

Often, acute toxicity is related to non-specific mode of actions, and not to mechanisms involving specific target molecules. The compounds are thought to interact with cellular membranes leading to unspecific membrane toxicity. This general mechanism may be only one, additional ones (e.g. oxidative stress) come into play with particular pharmaceuticals. We evaluated whether the acute toxicity data of the different classes of pharmaceuticals correlate with the log K_{ow} of the compound, as the lipophilicity determined by log K_{ow} is an important parameter for membrane toxicity. However, no correlation was found between the log K_{ow} of pharmaceuticals of a certain category or of all pharmaceuticals, and the acute toxicity either of a certain species, a group of organisms, or all of them. The best relation between measured and estimated log K_{ow} of one class of pharmaceuticals and acute toxicity in one species, *Daphnia magna*, is depicted in Figure 3. Reasons for the variability of the data are probably based on laboratory differences, nominal concentration differences, clone susceptibility differences, but also on the fact that log

K_{ow} may not be the best model for lipophilicity. This holds in particular for ionizable compounds, where the pH-dependent speciation is of significant influence (Brüschweiler *et al.* 1995; Looser *et al.* 1998).

In conclusion, acute toxicity to aquatic organisms is unlikely to occur at measured environmental concentrations, as acute effects concentrations are 100 to 1000 higher than residues found in the aquatic environment. For example, the lowest acute effect concentration of fluoxetine was 20 µg/L, whereas the highest estimated environmental concentration was 0.01 µg/L; the lowest acute effect of salicylic acid was 37 mg/L, whereas the highest environmental concentration was ~60 µg/L. Therefore, acute toxicity is only relevant in case of spills.

Chronic effects

Many aquatic species are continuously exposed over long periods of time or even over their entire life cycle. Evaluation of the chronic potential of micro-pollutants, e.g. pharmaceuticals, is therefore important. However, there is a lack of chronic data, and where available, chronic toxicity is marginally known. The available chronic data do often not investigate the important key targets, nor do they address the question in different organisms. Toxicity experiments are usually performed according to established guidelines. More specific investigations including analysis of possible targets of the pharmaceutical, or over different life stages, are lacking, or have only rarely been performed. Moreover, life cycle analyses are not reported, except for EE2 (Länge *et al.* 2001; Parrott and Blunt 2005), and toxicity to benthic and soil organisms have very

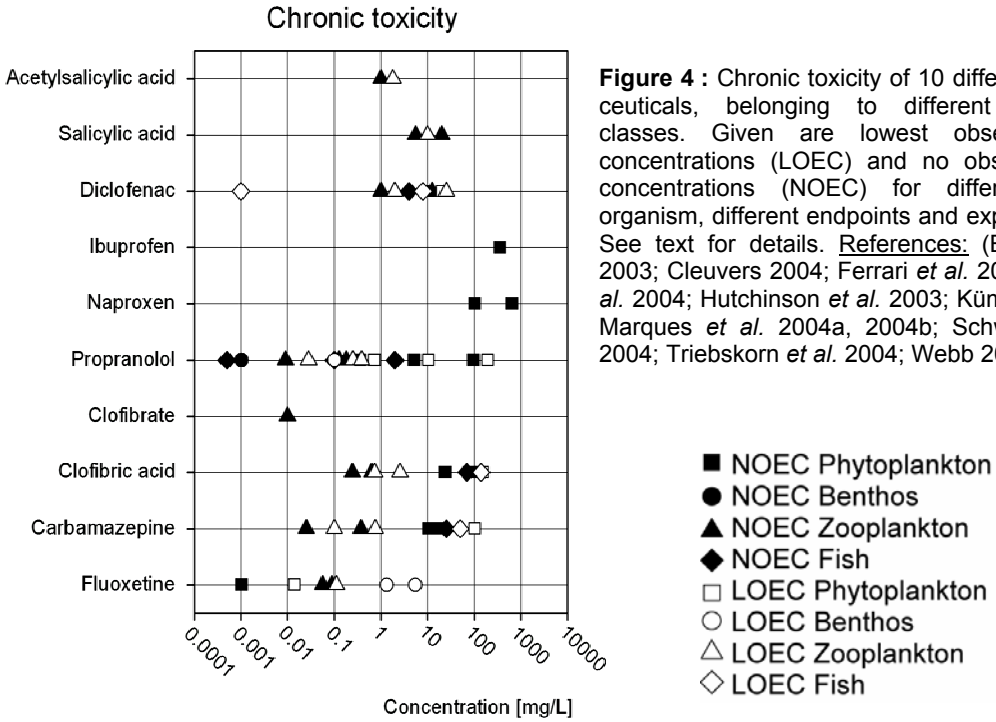


Figure 4 : Chronic toxicity of 10 different pharmaceuticals, belonging to different therapeutic classes. Given are lowest observed effect concentrations (LOEC) and no observed effect concentrations (NOEC) for different aquatic organism, different endpoints and exposure times. See text for details. References: (Brooks *et al.* 2003; Cleuvers 2004; Ferrari *et al.* 2004; Henry *et al.* 2004; Hutchinson *et al.* 2003; Kümmerer 2004; Marques *et al.* 2004a, 2004b; Schwaiger *et al.* 2004; Triebskorn *et al.* 2004; Webb 2001).

rarely been evaluated. In this chapter, we review the current literature according to the different pharmaceutical classes and summarize the data in Figure 4.

The best knowledge exists for the synthetic steroid EE2 contained in contraceptive pills, showing estrogenic effects at extremely low and environmentally relevant concentrations. This steroid has been shown in many fish to induce estrogenic effects at extremely low concentrations. Exposure of fathead minnows over their life cycle indicates reproductive effects at low concentrations of EE2 (Länge *et al.* 2001). The NOEC values of the F_0 generation F_1 embryo hatching success and larval survival were ≥ 1 ng/L. Male fish exposed to EE2 at 4 ng/L failed to develop normal secondary sexual characteristics and the sex ratio was altered. No testicular tissue was observed in any fish exposed to EE2 at 4 ng/L. A recent study shows vitellogenin (VTG) induction in fathead minnows with an EC_{50} value as low as 1 ng/L; EE2 was 25-30 times more potent than estradiol (Brian *et al.* 2005), confirming previous reports on VTG induction at concentrations between 0.1 and 1 ng/L (Pawlowski *et al.* 2004). Decreased egg fertilization and sex ratio (skewed toward females), both of which were significantly affected at extremely low concentrations of 0.32 ng/L EE2 (Parrott and Blunt 2005). The next most sensitive parameter was demasculinization (decreased male secondary sex characteristic index) of males exposed to an EE2 concentration of 0.96 ng/L. Full life cycle exposure of zebrafish to 3 ng/L EE2 lead to elevation of VTG and caused gonadal feminization in all exposed fish and thus inhibited reproduction (Fenske *et al.* 2005). Life-long exposure of zebrafish to 5 ng/L in the F_1 generation caused a 56% reduction in fecundity and complete population failure with no fertilization. Infertility in the F_1 generation was due to disturbed sexual differentiation with males having no functional testes and intersex gonads (Nash *et al.* 2004).

In hazard and risk assessment, the ratio between acute to chronic toxicity is often taken for evaluation of chemicals. For pharmaceuticals, this is difficult, because only very rarely, a systematic analysis of a given drug in both acute and chronic toxicity in a single species is performed. Apart from EE2, there are only a few NSAID, from which acute to chronic ratios can be deduced. Table 3 shows that even for similar drugs, these ratios in *Daphnia magna* vary by

Table 3
Ratio between acute and chronic toxicity in Daphnia magna and Ceriodaphnia dubia(48 h/21days)

Drug	Acute (mg/L)	Chronic (mg/L)	Ratio
Acetylsalicylic acid	1293.1	1.4	924
Salicylic acid	1031.7	13.3	77
Clofibrate	28.2	0.01	2820
Naproxen	66.4	0.33	201
Naproxen Na	43.6	0.68	64

Data after Marques et al. (2004a,b) (acetylsalicylic acid and salicylic acid, D. magna), Webb (2001) (clofibrate, D. magna) and Isidori et al. (2005) (naproxen and naproxen Na, Ceriodaphnia dubia).

two orders of magnitude. For all other drugs, only partial information is available on a given species. Ratios derived on the basis of a number of different species are not accurate, giving questionable information. The examples in table 3 confirm again that chronic toxicity cannot be derived from acute toxicity by simple calculations. This is often neglected in risk assessment.

Analgesics and non-steroidal anti-inflammatory drugs. NSAID inhibit the synthesis and release of prostaglandins via COX inhibition and these compounds are the most consumed category of drugs. About NSAID commonly found in the aquatic environment, most chronic data are reported. Acetyl salicylic acid affected reproduction in *D. magna* and *D. longispina* at concentrations of 1.8 mg/L (Marques *et al.* 2004a). Diclofenac is commonly found in wastewater at median concentration of 0.81µg/L (Daughton and Ternes 1999) whereas the maximal concentration in wastewater and surface water is up to 2µg/L (Daughton and Ternes 1999; Schwaiger *et al.* 2004; Stumpf *et al.* 1996). Traditional chronic toxicity studies with diclofenac were reported in invertebrates (Ferrari *et al.* 2004). A recent study demonstrated chronic histopathological effects in rainbow trout after 28 days of exposure. At the LOEC of 5 µg/L renal lesions (degeneration of tubular epithelia, interstitial nephritis) and alterations of the gills occurred in rainbow trout (Schwaiger *et al.* 2004), and subtle subcellular effects even at 1 µg/L (Triebskorn *et al.* 2004). Impairment of renal and gill function is likely to occur after long-term exposure. The kidney was also found to be a target of diclofenac in vultures, acute renal failure was probably the reason for the visceral gout (Oaks *et al.* 2004) and the occurrence of extensive deposits of uric acid on and within internal organs (Oaks *et al.* 2004). In zebrafish embryos, no effect of diclofenac on embryonic development was observed, except delayed hatching at 1 and 2 mg/L (Hallare *et al.* 2004). Additional side effects of diclofenac have been observed in humans in the liver with degenerative and inflammatory alterations (Banks *et al.* 1995), in lower gastrointestinal tract and in the esophagus (Bjorkman 1998), but not in fish.

Beta-blockers. As fish contain β_2-receptors in heart and liver (Gamprel *et al.* 1994) and probably in reproductive tissues (Haider and Baqri 2000), unspecific antagonists such as propranolol may be active in fish. In fact, propranolol indicated chronic toxicity not only on the cardiovascular system, but also on reproduction. The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) of propranolol affecting reproduction in *C. dubia* were 125 and 250 µg/L, and reproduction was affected after 27 days of exposure in *Hyalella azteca* at 100 µg/L (Hutchinson *et al.* 2003). In fish *O. latipes*, significant changes in plasma steroid levels occurred after 14 days of exposure. The number of eggs released by fish was reduced at 0.5 µg/L after a 4-week exposure to 0.5 and 1 µg/L, but not at 50 and 100 µg/L (Hutchinson *et al.* 2003). No alteration in vitellogenin levels was observed. It was suggested that alteration in sex steroids let to decreased oxytocin excretion, which could decrease the number of eggs released. Propranolol was also analysed in invertebrates. LOEC and NOEC for different organisms span several orders of magnitude (Figure 4), partly due to differences between

laboratories, but also species differences. These data should be compared to the environmental concentrations; propranolol, metoprolol and nadolol were identified in U.S. wastewater samples up to 1.9, 1.2 and 0.36 µg/L, respectively (Hutchinson *et al.* 2003).

Blood lipid lowering agents. Data on this class of compounds are rare. Fibrates have been evaluated by traditional toxicity tests. The following NOEC were found for clofibric acid in *C. dubia* [NOEC (7 days) = 640 µg/L], the rotifer *B. calyciflorus* [NOEC (2 days) = 246 µg/L], and in early life stages of zebrafish [NOEC (10 days) = 70 mg/L] (Ferrari *et al.* 2004). Gemfibrozil occurred in blood plasma of goldfish after exposure over 14 days at 113-times higher levels than in water (bioconcentration factor of 113). Plasma testosterone was reduced by over 50% after exposure to 1.5 and 10 mg/L, as well as levels of steroid acute regulatory protein transcript in goldfish testes (Mimeault *et al.* 2005).

Neuroactive compounds. Most data were reported for the antiepileptic carbamazepine and serotonin reuptake inhibitors (SSRI), other neuroactive compounds were very rarely or not evaluated (Figure 4). Traditional toxicity tests showed chronic toxicity of carbamazepine in *C. dubia* [NOEC (7 days) = 25 µg/L], in the rotifer *B. calyciflorus* [NOEC (2 days) = 377 µg/L], and in early life stages of zebrafish [NOEC (10 days) = 25 mg/L] (Ferrari *et al.* 2004). Carbamazepine is considered carcinogenic in rats but is not mutagenic in mammalian cells. Sublethal effects occurred in *Daphnia* at 92 µg/L and the lethal concentration in zebra fish was 43 µg/L (Thaker 2005). In a study with the cnidarian *Hydra vulgaris*, diazepam was shown to inhibit polyp regeneration at 10 µg/L (Pascoe *et al.* 2003).

Most chronic studies focussed on SSRI. Serotonin is a neurotransmitter found in lower vertebrates and invertebrates, and SSRI may adversely influence the function of the nervous and associated hormonal systems of these organisms as well. Besides having important functions as a neurotransmitter, serotonin may directly act on the immune system, alters appetite, influences behavior and modulates sexual function. The role of serotonin in reproduction varies between different phyla and effects of SSRI as well. Fong (1998) found that SSRI (fluvoxamine, paroxetine) led to induction (at 10 nM to 100 µM) and fluoxetine to potentiation (at 5 µM, and if co-applied with 7-100 µM serotonin, but not at other concentrations) of parturition in fingernail clams. Fong (1998) found an induction of spawning in zebra mussels by fluvoxamine concentrations as low as 0.032 µg/L. Induction of mussel spawning point to an interference with serotonin action, as in invertebrates, serotonin may stimulate ecdysteroids, ectysone and juvenile hormone, responsible for controlling oogenesis and vitellogenesis (Nation 2002). A reproductive stimulation was also found in *D. magna* exposed to 36 µg/L fluoxetine for 30 days, and in *C. dubia* fecundity was increased at 56 µg/L (Flaherty *et al.* 2001), but reduced in another study (Brooks *et al.* 2003). An evaluation of 5 SSRI (fluoxetine, fluvoxamine, paroxetine, citalopram, sertraline) showed negative effects on *C. dubia* reproduction by

reduction of the number of neonates or brood per female after 7-8 days of exposure. For the most active compound, sertraline, the LOEC was 45 µg/L and the NOEC 9 µg/L (Henry *et al.* 2004). Fluoxetine has been detected in sewage and stream water at concentrations of 12 ng/L (Kolpin *et al.* 2002) and 99 ng/L (Metcalfe *et al.* 2003b), respectively.

In medaka (*O. latipes*), serotonin induced oocyte maturation (Iwamatsu *et al.* 1993), but a contrary action was reported in mummichog (*F. heteroclitus*) (Cerda *et al.* 1998). Serotonin was indicated to potentiate effects of gonadotropin-releasing hormone on gonadotropin release from the pituitary (Oaks *et al.* 2004). When medaka were exposed for 4 weeks to fluoxetine concentrations of 0.1-5 µg/L, vitellogenin plasma content, plasma steroids, fecundity, egg fertilization or hatching rate were not affected (Brooks *et al.* 2003). This indicates no reproduction impairment in this fish up to 5 µg/L fluoxetine. Taken together the chronic effects of SSRI on reproduction of fish and invertebrates are not yet clear, interference with reproduction occurred at much higher concentrations than measured in surface waters.

Chronic data on various other compounds are lacking, although they have been shown to occur in considerable amounts in surface waters (Figure 2). This holds in particular for fish. For the anticancer compound, tamoxifen, chronic data are found for *Acartia tonsa* [EC50=49 µg/L, (Andersen *et al.* 2001)]. Various morphological and developmental effects (early embryonic mortality) were induced in sea urchin embryos after exposure to 10^{-8} to 10^{-5} M tamoxifen, which corresponded to oxidative stress. ROS production was increased and lead to oxidative damage and it is thought to represent a pro-oxidant mode of action explaining carcinogenicity in humans and rodents (Pagano *et al.* 2001).

The antiandrogenic compound flutamide and aromatase inhibitor fadrazole were also analysed for effects in fish, mainly as a positive controls for the evaluation of effects suspected for other environmental chemicals. Short-term reproduction assays in fathead minnows show that flutamide at 0.9 mg/L significantly reduced male sex characteristics in male fish. Fadrozole significantly inhibited ovarian growth and induced testis growth at 0.05 and 0.96 mg/L after 21 days, and inhibited VTG in females and induced VTG synthesis in males (Panter *et al.* 2004). Flutamide at 0.5 mg/L also reduced fecundity of the fish after 21 days. Embryo hatch was reduced and alterations in godadal histology were observed (Jensen *et al.* 2004). Ovaries from females indicated a decrease in mature oocytes and males exhibited spermatocyte degeneration and necrosis. Concentration-dependent VTG and testosterone increase were observed in females. Flutamide had an antiandrogenic effect and reduced fecundity, yet at rather high concentrations. Moreover, in adult male guppy, reduction in ejaculated sperms, reduced sex coloration and smaller testes occurred. The male courtship behaviour was also disrupted at 1 and 10 mg/kg in feed (Baatrup and Junge 2001). The aromatase inhibitor fadrozole reduced fecundity after 21 days at water concentrations of 10 and 50 µg/L and

inhibited brain aromatase activity (Ankley *et al.* 2002). In females a concentration dependent reduction in plasma estradiol and VTG was observed. In males, androgens in plasma were significantly increased and resulted in a marked accumulation of sperm in the testes.

In vitro studies

Several pharmaceuticals have been investigated in *in vitro* systems. They were mainly analyzed for acute cytotoxicity in fish cell lines and in primary fish cell cultures. Cytotoxicity of clofibrate, fenofibrate, carbamazepine, fluoxetine, diclofenac, and propranolol to the fish cell line PLHC-1 (hepatoma cells derived from topminnow) and primary cultures of trout hepatocytes was reported (Laville *et al.* 2004). Fibrates are known to enhance β-oxidation of lipids, which increases the amount of reactive oxidative species (ROS) in cells. Fenofibrate [EC_{50} (24 h) = 3.25 mg/L] and clofibrate [EC_{50} (24 h) = 0.46 mg/L] were the most active compounds (Laville *et al.* 2004). Cytotoxicity was higher in PLHC-1 than in primary hepatocytes. Oxidative stress is thought to be responsible for the cytotoxicity, at least for these fibrates (Laville *et al.* 2004). Cytotoxicity of fluoxetine [EC_{50} (24 h, PLHC-1) = 1.73 mg/L] was also mediated in part by oxidative stress. Besides cytotoxicity and ROS production, the pharmaceuticals were analyzed for their potential to induce cytochrome P4501A monooxygenase activity (CYP1A), which can be regarded as important for chronic toxicity. Among the tested drugs, the β-adrenergic receptor antagonist propranolol was the only CYP1A inducer in primary hepatocytes, the other 6 pharmaceuticals lead to inhibition of basal activity (Laville *et al.* 2004).

Furthermore, Henschel et al. (1997) evaluated the cytotoxicity of salicylic acid, paracetamol, clofibrinic acid and methotrexate to BF-2 fish cell line (fibroblasts derived from bluegill sunfish). For three out of four compounds the concentrations inducing *in vitro* cytotoxicity were lower as compared to *in vivo* studies with highly proliferating ciliates. The particular mode of action of methotrexate [EC_{50} (48 h) = 3 mg/L, (Henschel *et al.* 1997)] may negatively interact with cell proliferation and therefore survival. Sensitivity of cells to toxicants may vary within species, as demonstrated by a direct comparison between cytotoxicity on fish and rat cell lines (Rau *et al.* 2004), or depending on their origin, e.g. PLHC-1 cell lines are more sensitive than trout primary hepatocytes (Laville *et al.* 2004). Some of the differences may be based on the difference in the ability of the cells to metabolize toxicants. These *in vitro* studies indicate their usefulness for the acute toxicity evaluation, but also for investigations of the modes of action of pharmaceuticals including chronic toxicity parameters. Among the advantages of *in vitro* systems based on fish cells or reporter gene systems are their potential for screening and first evaluation of potential toxicity (Fent 2001). They are important alternatives to animal testing able to identify general toxicity and specific cellular targets and processes, and they are economic.

Toxicity of pharmaceutical mixtures and community effects

There are only a few studies dealing with the effects of mixtures of pharmaceuticals. Cleuvers (2003, 2004) has evaluated the ecological potential of antiinflammatory drugs and of diverse acting pharmaceuticals in different sets of biotests using different aquatic organisms. A mixture of NSAID (diclofenac, ibuprofen, naproxen, acetylsalicylic acid) has been evaluated using acute Daphnia and algal tests. Toxicity of the mixture was found at concentrations at which the single compound showed no or only little effects. The mixture toxicity followed the concept of concentration addition, which means that the concentrations of each compound behaved in an additive fashion.

Acute toxicity tests using *D. magna*, alga (*Desmodesmus subspicatus*) and macrophyte (*Lemna minor*) were performed to analyze for acute toxicity of 9 drugs having different modes of action (clofibric acid, carbamazepine, ibuprofen, propranolol, metoprolol, diclofenac, naproxen, captopril, metformin). The combined effects of two substances, clofibric acid and carbamazepine, followed the concept of concentration addition in the Daphnia test, whereas in the algal tests, the concept of independent action was adequate. When a combination of NSAID, ibuprofen and diclofenac, was analyzed, the effect on algae followed the concentration addition concept, whereas for Daphnia, the combination effect was stronger. These data indicate that for the acute toxicity of these pharmaceuticals, concentration addition can be assumed, which means that the concentration of each individual pharmaceutical has to be added for the combination effects. This implies that compounds occurring at concentrations below their individual NOEC can nevertheless contribute to the total effect of the mixture.

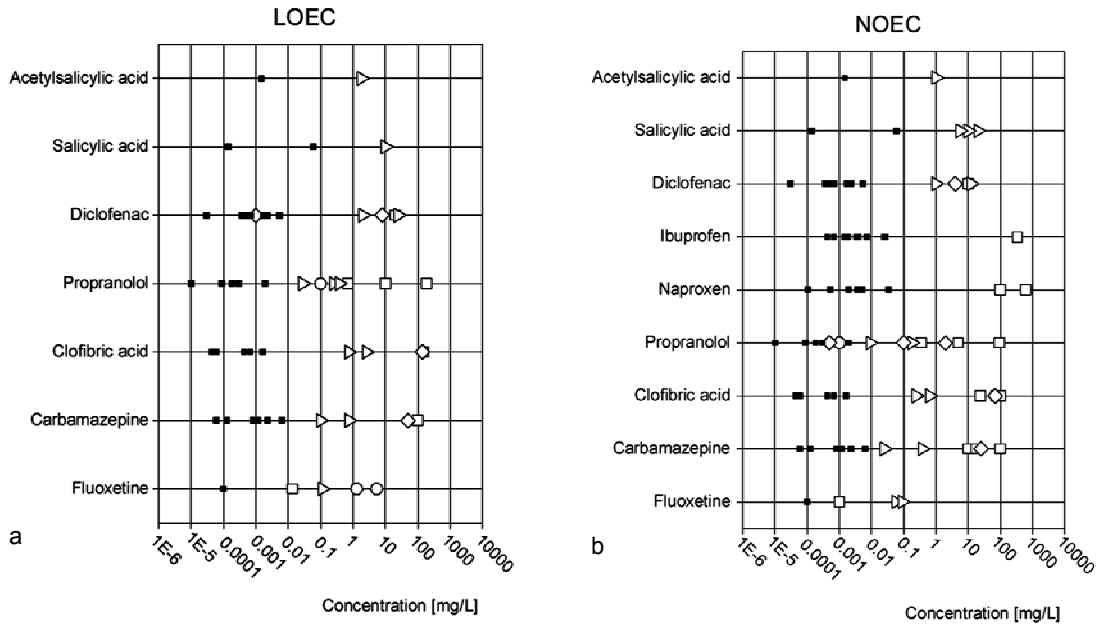
Only few pharmaceuticals have been analysed in ecologically more realistic model ecosystems, namely, microcosms and mesocosms. In two recent studies, outdoor aquatic microcosms of a total volume of 12’000 L containing water and sediment were used to analyze the effects of combination of pharmaceuticals. (Sanderson *et al.* 2004) evaluated the effects of combinations of 8 pharmaceuticals at 3 concentration levels on macrophytes *Lemnea gibba* and *Myriophyllum sibiricum* over a 35 days period. Atorvastatin, a blood lipid regulator, was among antibiotics the pharmaceutical eliciting phytotoxicity. Using similar microcosms effects on phyto- and zooplankton were assessed after exposure for 35 days at 3 concentrations to two pharmaceuticals (ibuprofen, fluoxetine) and an antibiotic (ciprofloxacin) (Brooks *et al.* 2003). The microcosms contained periphyton, phytoplankton, zooplankton, algae and benthic communities, and in addition, juvenile sunfish were exposed in mesh cages. Species abundance and number of phytoplankton and zooplankton were affected at the medium (60-100 µg/L each compound), and high treatment level (600-1000 µg/L each), whereas at the low treatment (6-10 µg/L each), only trends were visible, but no significant effects occurred. Unexpected high lethality occurred in fish at the high and medium treatments, lethality was observed in plants in addition to decreased growth. Decreased diversity of both phytoplankton

and zooplankton communities and increased abundance of both communities may have important ecological implications. However, the cause of the decline in diversity and the other effects was unclear (whether caused directly or indirectly and by what pharmaceutical having different modes of action). Maximal concentrations of ibuprofen, fluoxetine and ciprofloxacin detected in the U.S. were 1.0, 0.012 and 0.03 µg/L, respectively (Kolpin *et al.* 2002). Richards et al. (2004) concluded that a low probability exists that these three pharmaceuticals are currently present in surface waters at concentrations negatively affecting aquatic communities. By comparing calculated whole-body therapeutic doses – and not human and fish plasma levels - the authors note that all responses occurred at levels well below the equivalent pharmacologically active concentrations in mammals. Concentrations of pharmaceuticals in fish can reach significantly higher concentrations in plasma than in the ambient water (Mimeault *et al.* 2005).

Comparison of environmental concentrations and ecotoxicological effects concentrations

The potential risk of a substance to the environment is often characterized by comparing the Predicted Environmental Concentration (PEC) with the Predicted No Effect Concentration (PNEC). PEC of pharmaceuticals are often estimated using calculations, which include usage or

Figure 5: Comparison between maximal concentrations of pharmaceuticals in treated wastewater and their chronic toxicity in aquatic organisms. (a) Lowest observed effect concentrations (LOEC). (b) No observed effect concentrations (NOEC) for different aquatic organism, different endpoints and exposure times. References see Figure 1 (wastewater concentrations) and Figure 4 (chronic toxicity).



sales figures, population density, wastewater production and dilution in watersheds to generate likely concentrations in surface waters (Bound and Voulvoulis 2004; Halling-Sorensen *et al.* 1998; Jones *et al.* 2002; Sanderson *et al.* 2004; Straub 2002). Due to the lack of experimental data (in particular chronic) in the public domain on the ecotoxicity of pharmaceuticals, estimation of PNEC, and therefore hazard and risk assessment, is difficult or even impossible to perform. In the open literature or databases, for less than 1% of pharmaceuticals data are available, and only a small number of new pharmaceuticals have been undergone risk assessment using ecotoxicological tests (Halling-Sorensen *et al.* 1998; Jones *et al.* 2002; Sanderson *et al.* 2004) In the absence of experimental data, information is often derived from Quantitative Structure-Activity Relationships (QSAR) predictions, for example by applying the EPA's ECOSAR program (Jones *et al.* 2002; Sanderson *et al.* 2004). While being a pragmatic approach for identifying hazards or prioritizing critical substances, this concept is not sufficiently precise for accurate hazard and risk assessments of pharmaceuticals.

Here, we summarize and compare the currently available empirical data in the open literature on maximal STP effluent concentrations with chronic LOEC and NOEC concentrations of individual pharmaceuticals (Figure 5). This approach is based on experimental data allowing to prioritize pharmaceuticals according to their ecotoxicological potential and to gain knowledge about the worst case situation. As can be deduced from Figure 5, LOEC and NOEC values of the pharmaceuticals for different aquatic organisms are about one to two orders and two orders of magnitude, respectively, higher than maximal concentrations in STP effluents. For diclofenac, the LOEC for fish toxicity was in the range of wastewater concentrations, whereas the LOEC of propranolol and fluoxetine for zooplankton and benthic organisms were near to maximal measured STP effluent concentrations. This shows that for diclofenac, propranolol and fluoxetine the margin of safety is narrow, and chronic effects at highly contaminated sites can not be completely ruled out, in particular, when the combined effects of pharmaceutical mixtures are taken into account. However, median sewage effluent concentrations are lower and dilution in receiving waters result in lower levels in surface waters reducing the environmental risk. It should be noted, however, that more experimental data on chronic toxicity and on the bioaccumulation potential is needed to fully judge the environmental risk posed by individual pharmaceuticals.

Discussion

Pharmaceuticals have been tested in traditional ways. A set of mainly acute toxicity tests using traditional species such as an algae (mainly *Scenedesmus quadricauda*), zooplankton (*Daphnia magna*) and fish (species according to OECD guidelines) has been performed. In general, only

very few pharmaceuticals have been assessed for acute and chronic toxicity in fish. Moreover, only a few pharmaceuticals have been analyzed for chronic toxicity, again in the traditional way according to guidelines (OECD, US EPA). Based on these studies, no one would probably have been able to anticipate the current population decline of three species of vultures due to diclofenac exposure. Furthermore, these tests alone are not sufficient for deriving an accurate profile of the possible hazards and risks of the pharmaceutical in question. Current tests cover only a small set of laboratory organisms, which are often not sensitive enough and often not able to unravel adverse effects of pharmaceuticals. As a consequence, more specific tests are needed. Only chronic toxicity investigations using more specific toxicity parameters will lead to a more meaningful ecological risk assessment. The following working hypotheses should be addressed in future ecotoxicological investigations:

1. Pharmaceuticals as biologically active compounds may have similar (chronic) effects in non-mammalian animals (and even plants) as in mammals as target receptors and/or biomolecules are similar and conserved during evolution. Therefore, similar adverse (chronic) effects as in humans and mammals may occur in lower vertebrates and invertebrates.
2. Some pharmaceuticals may have unexpected (chronic) effects in lower organisms due to biological differences in pharmacodynamics, pharmacokinetics and physiology.
3. *In vitro* studies of pharmaceuticals are important for screening, elucidating the modes of action in non-target organisms, and designing specific *in vivo* studies.

One approach to address these hypotheses is to include histopathological investigations in chronic fish toxicity studies. By focusing on specific tissues and organs, more detailed answers about possible adverse effects may be obtained. This is exemplified by a study on chronic effects of diclofenac in fish (Schwaiger *et al.* 2004; Triebskorn *et al.* 2004). Another approach is to use the existing knowledge about possible side effects of the compound of interest in mammals and humans for the design of specific analysis in aquatic organisms. Furthermore, known drug-drug interactions in humans may be relevant for compound mixtures in the environment. Both are based on the hypothesis that targets of the pharmaceutical may be identical or similar in lower organisms as receptors, biochemical pathways and enzymes are conserved in evolutionary terms. This holds true for nuclear steroid receptors that are very similar in organisms of different evolutionary levels (Sanderson *et al.* 2004), nuclear peroxisome-proliferator activated receptors (PPAR's) (Escriva *et al.* 1997), adrenoceptors such as β_1- and β_2-receptors (Nickerson *et al.* 2001), but also for insulin receptor, insulin-like growth factor and glucagon receptors being present in lower vertebrates and invertebrates (Villegas-Navarro *et al.* 2003). Also, basic mechanisms like signal transduction, cell division, and key metabolizing enzymes such as cytochrome P450s are conserved in a large variety of organisms

(Nelson *et al.* 1996). As a consequence, analysis of pharmaceuticals should specifically be directed to

- specific and identical targets (biomolecules, tissues, organs): target specificity
- known adverse side effects in humans and mammals: side effect specificity
- general chronic effects for accounting physiological differences: species specificity

Our proposed strategy for future research on the ecotoxicology of pharmaceuticals is exemplified by a few examples. When the ecotoxicity of NSAID is studied, effects on inhibition of prostaglandin synthesis and COX inhibition should be addressed in lower organisms, and at the same time, on side effects already known in mammals. Diclofenac has been known for causing side effects on the kidney (and other organs such as liver) in mammals, subsequently being found in vultures (Oaks *et al.* 2004), and fish (Schwaiger *et al.* 2004). Cardiovascular pharmaceuticals should be analyzed for their possible effects on the cardiovascular system in lower vertebrates. Lipid lowering agents such as fibrates are also known to act by enhancing or reducing PPAR (Kliewer *et al.* 1997). These nuclear receptors play key roles in the catabolism and storage of fatty acids and are important for blood lipid regulation. Indeed, PPAR's are affected in amphibians (Kliewer *et al.* 1997) and fish by clofibrate, benzafibrate and fenofibrate (Ruyter *et al.* 1997).

Beta-blockers bind to the beta-adrenergic receptors and block its activation by physiological agonists. These receptors are located in mammals in many tissues including heart, and its blockade causes a decrease in heart rate and contraction. Beta-blockers differ in specificity to the different receptor subtypes, some are non-specifically acting on β_1- and β_2- receptors (e.g. propranolol), while others are specific for the β_1-receptor subtype (e.g. atenolol). In *D. magna*, heart beat rate, fecundity and biomass were reduced after chronic exposure to 0.11 mg/L (Dzialowski *et al.* 2003), although it is not know whether β_2-receptors occur. Long-term exposure to propranolol reduced reproduction in *C. dubia* at 250 µg/L and in *H. azteca* at 100 µg/L (Hutchinson *et al.* 2003).

A class of anti-hyperlipidemic drugs inhibit their target enzyme hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) in mammals (Seiler 2002). Whether these enzymes are also inhibited in lower animals should be addressed in future investigations. Surprisingly, atorvastatin was even found in a plant (duckweed, *Lemna gibba*) to have effects, but the mode of action is unclear (Sanderson *et al.* 2004).

Many antineoplastic drugs used in cancer therapy have a high mutagenic and cancerogenic potential. Parent compounds are often bioactivated leading to formation of mutagenic metabolites (e.g. cyclophosphamide, ifosfamide). In case organisms in the environment are able to metabolize these pharmaceuticals, enhanced mutation frequencies and cancer risk will result.

In addition, these drugs often have significant side effects on humans such as nausea, cytotoxicity, reduction in proliferation of cells in various tissues etc. One would expect mutagenicity and cancerogenicity to occur in exposed aquatic organisms as well. However, such studies are lacking besides the analysis of hospital wastewater, in which the genotoxic potential was based on antibiotics such as ciprofloxacin (Hutchinson *et al.* 2003).

It should be noted, however, that besides known targets additional or other target tissues and organs may be affected alternatively. This would result in unexpected effects not targeted by the investigations. Examples are effects on sex hormones in blood plasma of fish and reduced reproduction in *C. dubia* and *H. azteca* induced by the beta-blocker propranolol after long-term exposure (Hutchinson *et al.* 2003), and the effects of serotonin-reuptake inhibitors on reproduction of mollusks (Chong *et al.* 2000). As the effects of the antiestrogen tamoxifen indicate, pharmaceuticals may have not only one, but multiple modes of action, such as oxidative damage in addition in case of tamoxifen (Pagano *et al.* 2001). This fact complicates the strategy to analyze for chronic effects. However, many of these unexpected chronic responses will be elucidated in the context of careful chronic toxicity analyses including histopathology and reproduction. However, such analyses are more expensive and probably only justified for important pharmaceuticals occurring in significant concentrations in the environment. But in the light of the limitations of traditional (acute) toxicity testing for use in environmental risk assessment, more specific toxicity analyses should be performed in forthcoming studies, taking full advantage of the available knowledge that is generated during the pharmaceutical drug development process (e.g. mechanisms of action, pharmacokinetic behaviour and metabolism, target organs and side effects in mammals).

In vitro studies are important for screening and evaluation of possible cellular targets in ecotoxicology (Fent 2001). They are also important in the reduction of animal experiments, in conjunction with other proposed new strategies (Hutchinson *et al.* 2003). Effects of pharmaceuticals have been evaluated in fish primary cells and fish cell lines indicating this potential (Laville *et al.* 2004). We assume that investigating pharmaceuticals in *in vitro* systems will not only allow a reduction of animal experiments, but also a better and more accurate characterization of possible targets of pharmaceuticals. These test systems not only allow the analysis of specific receptor interactions and target enzymes in animal and plant cells, but also a rapid screening of a large number of compounds.

Pharmaceuticals are analyzed for possible ecotoxicological effects as single compounds and only rarely as mixtures (Cleuvers 2003). However, as other environmental pollutants pharmaceuticals are present in the environment in mixtures. Effects of mixtures most probably follow the concept of concentration addition, hence, the overall toxicity is the result of the sum of the individual concentration of each compound. Therefore effects may occur even at the NOEC

of individual compounds. It should also be recognized that even subtle changes of normal homeostasis including behavioral alterations may have direct and indirect effects, even if only minor ones, that eventually result in significant deteriorating effects on a species or population in the ecological context. The extreme case of the dramatic poisoning and population declines of Indian vultures is a case in point. The dimension of this population decline has no parallel in birds since the disappearance of peregrine falcons and other predatory birds in the 1960s due to the pesticide DDT.

Conclusions and future directions

One important aspect to solve the load of pharmaceutical residues in wastewater and surface water is to optimize STP processes. There is a need to increase the knowledge about the fate of pharmaceuticals during sewage treatment for implementation of better removal techniques. Future work on STP treatment optimization will show to what extend pharmaceuticals can be removed from wastewater and to what extent the implementation of an improved technology is feasible, taking into account other macro- and micro-pollutants as well as the broad variety of complex wastewater matrices.

Our present knowledge about residues of pharmaceuticals in aquatic systems indicate that they are unlikely to pose a risk for acute toxicity. Environmental concentrations are in the range of 10^3- 10^7-times lower than known LC_{50} or EC_{50} values (ratio of 10^3 between lowest acute effect of fluoxetine and highest environmental concentration; difference of 10^7 between highest LC_{50} of diazepam and highest environmental concentration). However, as the collapse of vulture populations in the Indian subcontinent indicates, important adverse effects can occur under certain circumstances.

There is a general lack of chronic toxicity data on pharmaceuticals, in particular in fish. Many pharmaceuticals need more investigation about potential long-term ecotoxicological effects, particularly with respect to potential disturbances in hormonal homeostasis (endocrine disruption), immunological status, or gene activation and silencing during long-term exposure. For better understanding of possible effects, a mechanism-based approach focused on target molecules, tissues and organs should yield more meaningful results and insights than traditional acute toxicity testing. Current data on acute and chronic toxicity of pharmaceuticals support to the conclusion that more target- or biomolecule-oriented, or mode-of-action-based investigations, will allow more relevant insights into effects on survival, growth and reproduction than traditional standard ecotoxicity testing. Often, similar target biomolecules are present in non-mammalian organisms and so are the adverse effects. *In vitro* systems are very important

tools for both elucidating modes of action in lower vertebrates, and for screening of the ecotoxicological potential of pharmaceuticals prior to fish toxicity testing. Unless more is known about possible chronic effects of individual pharmaceuticals and mixtures thereof, conclusions concerning hazards or risks of pharmaceuticals to the aquatic ecosystem are premature.

Drugs may also induce unexpected effects in non-mammalian organisms, however. This is based on the difference in pharmacokinetics and pharmacodynamics, important parameters for occurring species differences. Disturbances of the reproductive system and hormone system, immune depression, neurobehavioral changes, to name some key targets, may have far reaching effects on the population level. This has become evident for endocrine disrupters such as steroid hormones used in contraceptives resulting in important adverse effects at environmentally relevant concentrations (Jobling *et al.* 1998; Länge *et al.* 2001; Parrott and Blunt 2005; Thorpe *et al.* 2003).

Comparison of available chronic toxicity data with environmental concentrations indicate that for most investigated pharmaceuticals concentrations are too low in aquatic systems to induce chronic effects on traditional laboratory organisms such as inhibition of algal growth and reproduction in *Daphnia*. For diclofenac, the LOEC for fish toxicity on an organ level was in the range of wastewater concentrations, however (Schwaiger *et al.* 2004), whereas the LOEC of propranolol and fluoxetine for zooplankton and benthic organisms were near to maximal measured STP effluent concentrations. Whether or not the margin of safety is narrow for additional human pharmaceuticals should be investigated in future studies. The future requirement of chronic testing with algae, daphnids and fish instead of only traditional acute toxicity studies is an important step forward (EMEA 2005). Moreover, the potential of combined effects of pharmaceutical mixtures should be addressed. In the ecological context, subtle changes and disturbances may have negative consequences for the organism's fitness. As a consequence much more should be known about the potential for chronic effects of pharmaceuticals in the aquatic system.

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APPENDIX 2

Estrogenic Activity of Pharmaceuticals and Pharmaceutical Mixtures in a Yeast Reporter Gene System

Karl Fent[a,b], Claudia Escher[a,b], Daniel Caminada[a,c]

[a] University of Applied Sciences Northwestern Switzerland, School of Life Sciences, Institute of Ecopreneurship, St. Jakobs-Strasse 84, CH-4132 Muttenz, Switzerland

[b] Swiss Federal Institute of Technology, Department of Environmental Sciences, CH-8092 Zürich, Switzerland

[c] University of Zürich, Institute of Plant Biology, Limnology, Seestrasse 187, CH-8802 Kilchberg, Switzerland

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Abstract

Pharmaceuticals enter aquatic environments in unchanged form or as metabolites. Little is known about their potential hormonal activity, which is of particular interest due to potential long-term effects on fertility and reproduction in aquatic organisms. Moreover, there is a need to assess the combined activity of pharmaceutical mixtures. In this study, 37 pharmaceuticals have been analysed *in vitro* for estrogenic activity using a recombinant yeast system expressing the human estrogen receptor α. Six pharmaceuticals belonging to different therapeutic classes, cimetidine, fenofibrate, furosemide, paracetamol, phenazone and tamoxifen, exhibited weak estrogenic activity. Furosemide showed an almost full concentration-response curve, whereas the other compounds showed low efficacy. The half-maximal activities of the pharmaceuticals were in the range of 0.66-25.53 mM. Furthermore, binary mixtures of furosemide and 17β-estradiol (E2), and furosemide and phenazone, and mixtures of up to five active pharmaceuticals were assessed for their combinatory activity at different equipotent concentrations. The estrogenic activity of binary mixtures of furosemide with E2 and phenazone, respectively, followed the model of concentration addition (CA). Mixtures of other pharmaceuticals often deviated from the CA model, because extrapolations become inaccurate with only partial and non-parallel concentration-response curves having low efficacy. This demonstrates that full and parallel concentration-response curves are a prerequisite for accurate predictions of mixture activity. Our study demonstrates for the first time weak estrogenic activity *in vitro* of some common pharmaceuticals and their mixtures.

Key words: Pharmaceuticals; Estrogenic activity; Mixture activity; Ecotoxicological effects; Environmental toxicology; Endocrine disrupters

Introduction

Pharmaceuticals are becoming increasingly important as potential environmental contaminants due to their widespread presence in aquatic ecosystems. Being designed for human or veterinary medicine, they may be of concern because of potential adverse effects on organisms in the environment. Pharmaceuticals have a specific mode of action that makes them effective in the therapeutic use at relatively low concentrations. Even though aquatic organisms lack some of the target receptors for pharmaceuticals, adverse effects can still occur via unspecific action (Fent *et al.* 2006). Currently, only very little information is available on acute and chronic effects despite aquatic organisms may be exposed to pharmaceuticals during their whole life span. As the ecotoxicological potential of pharmaceutical residues remains almost unknown (Daughton and Ternes 1999; Fent *et al.* 2006; Laville *et al.* 2004), it is necessary to evaluate the potential ecological risks of pharmaceutical residues in particular with respect to their hormonal activity, which may have consequences on the population level.

The exposure of aquatic organisms to pharmaceuticals and their metabolites may be significant in view of the often considerable levels occurring in treated effluents of sewage treatment plants (STPs), contaminated waters (Fent *et al.* 2006; Halling-Sorensen *et al.* 1998; Kolpin *et al.* 2002; Kümmerer 2004; Ternes *et al.* 2004) and landfill leachates (Fent 2004). Widely used pharmaceuticals are found in the ng/L to µg/L range in municipal wastewaters (Fent *et al.* 2006; Kümmerer 2004; Ternes *et al.* 2004), which also holds for certain antibiotics (Hartmann *et al.* 1998; Heberer 2002; Kümmerer 2003). Pharmaceutical residues constitute a chronic exposure of wildlife at discharge sites (Ashton *et al.* 2004).

Even in concentrations near the detection limit natural and synthetic sex hormones can cause adverse effects. At environmentally relevant concentrations 17β-estradiol (E2) and the synthetic 17α-ethinylestradiol (EE2) leads in fish to induction of the yolk precursor vitellogenin, effects on gonadal histology, reduction of fecundity and reproductive success (Cromlish and Kennedy 1996; Sumpter and Johnson 2005). The *in vivo* potency of EE2 is 10 to 50-fold higher than that of E2 (Cromlish and Kennedy 1996; Thorpe *et al.* 2003), probably due to lower metabolism. Already 0.1 ng/L EE2 induces the expression of vitellogenin in fish (Purdom *et al.* 1994), 0.1-15 ng/L can affect sex differentiation (Van Aerle *et al.* 2002), and 2-10 ng/L may affect fecundity negatively (Sacher *et al.* 2001). Life-long exposure to 5 ng/L leads to significant

reduction in fecundity in the F1 generation and complete population failure due to lack of fertilization (Nash *et al.* 2004). Thus, given its concentration in the environment frequently being between 0.5 and 7 ng/L, EE2 may be a significant contributor to reproductive dysfunction in wild fish. Besides sex hormones, a rising number of natural and man-made substances are able to disturb the endocrine and reproductive system of organisms by mimicking hormone action (Damstra *et al.* 2002) due to their binding to the estrogen receptors (Rajapakse *et al.* 2004). Among them are widely used compounds such as UV-filters (Kunz *et al.* 2004), bisphenol A (Vom Saal and Hughes 2005) or phthalates (Swan *et al.* 2005) to mention a few. So far, pharmaceuticals have not been reported to have hormonal effects.

Current methods of risk assessment usually focus on the assessment of single chemicals. This is in contrast to the exposure situation in aquatic ecosystems, where organisms are typically exposed to a variety of estrogenic compounds. Alone, these concentrations are rather unlikely to cause estrogenic effects with the exception of EE2 and E2 (Sumpter and Johnson 2005). However, when acting together they can pose a hazard, which may be underestimated by focusing on individual compounds alone. It is difficult to assess the effects of a complex mixture of estrogenic substances (Kortenkamp and Altenburger 1998; Rajapakse *et al.* 2002; Rajapakse *et al.* 2004; Stumpf *et al.* 1999). Effects of mixtures cannot be calculated by simply adding the effects of the mixture components when applied alone, especially if the components have differently shaped dose-response curves (Payne *et al.* 2000). Two competing models are found widely spread in literature: the concept of concentration addition (CA) and the concept of independent action (IA) (Kortenkamp and Altenburger 1998). The CA concept is based on the assumption that the substances in a mixture have the same mode of action. Thus, one compound in a mixture can be replaced by another compound in a concentration that provokes the same respective effect. The effect of the mixture is therefore an addition of the effects of the single compounds in proportion to their relative fraction in the mixture. The IA model in contrast is applied when substances are involved that have a different mode of action. The effect of one compound is independent of the others. IA can therefore not be used, when the substances bind to the same receptor (Backhaus *et al.* 2004). By using a recombinant yeast system, mixtures of estrogenic compounds were shown to interact *in vitro* according to the CA model (Kortenkamp and Altenburger 1998; Rajapakse *et al.* 2002). The components in the mixture contribute to the overall effect by acting in relation to their potency, even at low-effect concentration below the threshold of detectable effects

(Stumpf *et al.* 1999). By using vitellogenin induction, the validity of the CA concept has also been demonstrated for mixture effects *in vivo* in fish (Cromlish and Kennedy 1996). The estrogenic chemicals had the capacity to act together in an additive manner. Thus far, only very few estrogenic chemicals have been analysed for mixture activity, and none of the pharmaceuticals besides EE2.

The compounds investigated in this study come from various therapeutic classes and have been chosen on the basis of its widespread use and potential hormonal activity. Nine pharmaceuticals can be assigned to the class of non-steroidal anti-inflammatory (NSAID)/analgesic/antiphlogistic drugs. Acetylsalicylic acid, ibuprofen, paracetamol, naproxen, and diclofenac are very often detected in sewage effluents and surface waters up to µg/L (Daughton and Ternes 1999; Fent *et al.* 2006). Rofecoxib is the active ingredient of Vioxx®, an anti-inflammatory agent. Five beta-blockers are considered as some of them were determined in wastewater (Fent *et al.* 2006). Antidepressants (fluoxetine, diazepam) and the antiepileptic carbamazepine, which is often found in wastewaters in concentrations of up to 2 µg/L (Gagne *et al.* 2005) are also considered. The blood-lipid lowering agents analysed in our study include seven statins and fibrates. Fenofibrate and clofibrate are widely used (Laville *et al.* 2004). Clofibric acid is the active metabolite formed via hydrolysis in organisms and the aquatic environment (Daughton and Ternes 1999). Gemfibrozil has been shown to have a potential for endocrine disruption in an *in vivo* study in goldfish (Mimeault *et al.* 2005). Two diuretics (furosemide and hydrochlorothiazide) have been analysed as well as the anti-estrogens tamoxifen and OH-tamoxifen. Sildenafil is the active ingredient of Viagra® and was considered because of its increasing use. In addition, adrenaline ((±)-epinephrine and (-)-epinephrine) have been analysed, and caffeine because of its ubiquity in the aquatic environment.

The aim of our study was to analyse a series of pharmaceuticals for their estrogenic activity employing a widely used yeast reporter gene system (Routledge and Sumpter 1996). To our knowledge, hormonal activity of pharmaceuticals occurring in the aquatic environment has not yet been reported. Generally, the potential estrogenic activity of pharmaceuticals remains elusive. As these substances are present in the environment as mixtures and at very low concentrations, the analysis of mixture effects is of particular interest. Our investigation demonstrates the estrogenic activity of five commonly used pharmaceuticals and the combined effect in mixtures. These *in vitro* data point to a further assessment of potential hormonal activity of pharmaceuticals in the environment.

Materials and Methods

Chemicals. The substances analysed in this study are listed in Table 1. A few pharmaceuticals were obtained as a gift; atorvastatin, diclofenac, metoprolol, simvastatin, carbamazepine, fluoxetine and atenolol were from Novartis International AG (Basel, CH), bezafibrate and diazepam were from F. Hoffmann-La Roche Ltd (Basel, CH). Doxorubicin, rofecoxib, sildenafil base were from Sequoia Research Products (UK) and pravastatin from ChemPacific (USA). The remaining pharmaceuticals and the solvent dimethylsulfoxide (DMSO) were all purchased from Sigma AG (Buchs, CH). Chlorophenol red-β-D-galactopyranoside (CPRG) was obtained from Roche (Basel, CH). Ethanol and methanol for glassware cleaning were obtained from J.T. Baker (The Netherlands). Stock solutions of pharmaceuticals in DMSO were prepared at standard concentrations of 500 mM. Where insoluble, stock solutions were diluted until the substance was dissolved.

Handling of recombinant yeast cells and YES-assay. Glassware was rigorously cleaned prior to use by washing twice with ethanol or once with methanol and once with ethanol. All work was carried out in a type II laminar flow cabinet. Glassware, test solutions, and assay medium were autoclaved after use for biosafety reasons. Recombinant yeast cells containing the human estrogen receptor (hERα) stably integrated into their main chromosome and expression plasmids carrying the reporter gene lac-Z were a gift from J. P. Sumpter, Brunel University, UK. Binding of an estrogen or xenoestrogen to the hERα in the yeast cell leads to the activation of the receptor controlling the expression of the reporter gene lac-Z. The enzyme β-galactosidase, which is then produced, is secreted into the assay medium containing the yellow CPRG, which is thereby metabolized into a red product that can be measured spectrophotometrically at 540 nm. Yeast stocks were stored in cryogenic vials at -20°C and renewed after a maximum of 4 months.

The YES-assay was carried out mainly as described by (Routledge and Sumpter 1996). 125 μL of yeast stock were incubated with approximately 50 mL of growth medium overnight at 28°C and 110 rpm on an orbital shaker. Serial dilutions of pharmaceutical stock solutions were prepared in DMSO and 5 μL aliquots of these solutions were transferred to 96-well optically flat-bottom microtiter plates. Assay medium was prepared by adding 0.5 mL CPRG and approximately $2x10^6$ yeast cells to 50 mL fresh growth medium. 195 μL of assay medium was then added to each well on

Table 1: Pharmaceuticals analysed

Therapeutic class	Pharmaceuticals	
Anti-inflammatories, analgesics, antiphlogistics	Diclofenac sodium Ibuprofen Mefenamic acid Naproxen Paracetamol	Phenacetine Phenazone Rofecoxib Salicylic acid
Beta-blockers	Acebutolol Atenolol (±)-Metoprolol	(±)-Propranolol HCl (S)-Propranolol HCl
Antiepileptics, antidepressants	Carbamazepine Diazepam	Fluoxetine
Blood lipid lowering agents	Atorvastatin Bezafibrate Clofibric acid Fenofibrate	Gemfibrozil Pravastatin Simvastatin
Various other compounds	Caffeine Cimetidine(H_2-receptor blocker) Doxorubicin HCl (cytostatic) (-)-Epinephrine (adrenaline, catecholamine) (±)-Epinephrine (adrenaline, catecholamine) Furosemide (diuretic) Hydrochlorothiazide (diuretic)	Metformin (antidiabetic) Methotrexate (cytostatic) Ranitdine (H_2-receptor blocker) Sildenafil base (PDE-inhibitor) Tamoxifen (antiestrogen) OH-tamoxifen (antiestrogen)

the plates. Each plate contained a row of 2.5% DMSO as control and a positive control of E2 ($2.5x10^{-6}$-$1.22x10^{-9}$ mM) in triplicate used as a standard. Plates were then sealed with microtiter plate sealers, incubated at 30°C and shaken every day for 2 min at approximately 200 rpm on a microtiter plate shaker. After 72 h, the plates were read using a microtiter plate reader (GENios, TECAN, CH) at an absorbance of 540 nm to detect colour change of the assay medium, and at 620 nm to measure turbidity as an indicator of yeast growth.

Data processing. Raw data were corrected for the DMSO controls and turbidity:

$$CorrectedAbsorbance = Chem.Abs(CPRG) - Chem.Abs(CPRG)_{Blank} - (Chem.Abs(YeastGrowth) - Chem.Abs(YeastGrowth)_{Blank}) \quad (1)$$

Some of the tested pharmaceuticals showed toxicity in the highest test concentrations and lead to cell lysis. As yeast growth in the wells is accompanied by turbidity, clear wells indicate a toxic pharmaceutical concentration. Corrected absorbance values from the plate reader are negative for wells containing toxic concentrations of test substances.

Screens for estrogenic activity of single substances were carried out twice using two replicates each. Substances showing no colour change in the assay medium were then

tested in quadruplicate, and the experiments were repeated twice. Pharmaceuticals showing induction were analysed in quadruplicates as well and experiments were repeated at least three times in order to get reliable concentration-response curves for mixture calculations.

Concentration-response curves for all substances were calculated using a non-linear regression (curve fit) model by GraphPad Prism® (GraphPad Software, San Diego, USA) software. The four-parameter logistic equation fit is expressed by the following equation:

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogEC_{50} - X) * HillSlope}} \qquad (2)$$

where *Y* is the response (absorption), *Top* the maximum and *Bottom* the baseline response respectively, $LogEC_{50}$ corresponds to the logarithm of the half-maximal effect concentration, *HillSlope* quantifies the steepness of the curve and is also called the slope factor.

Mixture experiments. We considered only pharmaceuticals for mixture experiments showing an induction of β-galactosidase expression. Compounds showing an anti-estrogenic effect in addition to the estrogenic effect were not included in mixture experiments, because estimates of mixture effects were not possible. All mixture assays were carried out identically to the single compound assay. The compound mixtures were diluted and transferred in 5 µL aliquots to the microtiter plates.

First, equipotent mixtures of E2 and furosemide were considered in various ratios. Subsequently, several equipotent mixtures of two to five active pharmaceuticals were carried out in different concentrations. In total 23 different mixture combinations were analysed. The concentrations of each compound in the mixtures were defined in

Table 2: Cytotoxic concentrations of pharmaceuticals in the YES-assay.

Cytotoxic concentration	Substances
Not detectable	Atenolol, caffeine, cimetidine, fenofibrate, metformin, methotrexate, paracetamol, phenazone, pravastatin, ranititdine, rofecoxib, salicylic acid, sildenafil, simvastatin
>1 mM	(+)-Epinephrine, (–)-epinephrine, acebutolol, bezafibrate, carbamazepine, clofibric acid, furosemide, gemfibrozil
>0.1 mM	Atorvastatin, diazepam, hydrochlorothiazide, ibuprofen, mefenamic acid, metoprolol, naproxen, phenacetine
>0.01 mM	(±)-Propranolol, (S)-propranolol, diclofenac
>0.001 mM	Doxorubicin, fluoxetine, OH-tamoxifen, tamoxifen

relation to the normalized effects obtained by E2; 25% of the maximal induction by E2 was labelled the C25 concentration, 20% of maximal induction, the C20 concentration etc. Equipotent mixtures of all respective compounds were analyzed up to a concentration of C10 for each compound. Higher concentrations were not analysed, as fenofibrate exhibiting the lowest estrogenic activity had an activity of 14% relative to E2.

Concentrations causing a 10 % increase in β-galactosidase expression range from 3.48 x 10^{-5} M (E2) up to 11.042 mM (paracetamol). The individual compounds were serially diluted in DMSO at a ratio of 1:1. For various pharmaceutical mixtures C10 and C01 concentrations were analysed. Furosemide and phenzone were mixed at C20 and C25, and four different binary mixture combinations at C01 and C10. Four mixture combinations each consisting of three pharmaceuticals were analysed at C10. A mixture of cimetidine, fenofibrate, furosemide and phenazone was analysed at C01 and C10, and a mixture consisting of these four compounds and phanazone at C01. Furthermore, a mixture of cimetidine, fenofibrate, furosemide and phenazone at the no observed effect concentration (NOEC) was analysed. The NOEC was estimated as the concentration of a compound where 0.1% of effect is reached (C0.1) via interpolation using the concentration-response curves (Table 3). The calculated concentration was divided by two, in order to achieve a C0.05 concentration, which is labelled as the NOEC.

Calculations for mixture effect estimations. Because none of the active pharmaceuticals showed a maximal response as the standard E2, it was not possible to mix the compounds at equipotent concentrations according to their effect concentrations (EC). First, a standard curve for E2 was established with ECs 1 to 99. A non-linear regression analysis using the four-parameter logistic equation performed with GraphPad Prism® for each estrogenic single substance then provided the concentrations at which each substance caused the respective absorptions. Thus, equipotent mixture concentrations could be calculated using an EC for E2 and a respective C value producing the same effect for single substances even for mixtures containing only substances producing a submaximal response or substances with non-parallel concentration-effect curves.

Two competing concepts frequently used for the prediction of mixture effects were taken into account: Concentration Addition (CA) and Independent Action (IA). CA is expressed by the following equation (Altenburger *et al.* 2000):

$$ECx_{Mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right) \qquad (3)$$

where ECx_{Mix} stands for the concentration of the mixture causing the x% effect and p_i is the fraction of the component *i* in the given mixture. The number of components *n* ranged from two to five in the conducted assays. ECx_{Mix} was calculated using CA equation in steps of 1 from 1 to the maximal C value that was reached by all the involved substances. Using the GraphPad Prism® software, the best-fit values for a sigmoidal dose-response curve with variable slope were calculated and the missing values for higher concentrations of the mixture were then extrapolated in order to get a full dose-response curve.

The mathematical formulation for the other considered concept of mixture prediction, IA is as follows (Altenburger *et al.* 2000):

$$E(c_{Mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \qquad (4)$$

Here, $E(c_{Mix})$ stands for the predicted effect of the mixture, c_i is the concentration of the *i*th compound and $E(c_i)$ is the effect of this concentration if the compound was applied separately. Again, the IA curve was calculated as high as practicable and then extrapolated using the sigmoidal dose-response (variable slope) curve as above.

Statistics. A one-sample t-test was used to compare the mean of values derived from the mixture experiments to the estimated mean calculated using the CA or the IA concept, respectively.

Results

Estrogenic activity of single compounds

At high concentrations, cytotoxicity was indicated in yeast cells when corrected absorbance values were lower than zero. This occurred at high levels with 24 pharmaceuticals (Table 2). In case cytotoxicity occurred, the stock solution of the pharmaceutical was diluted prior to preparation of the serial dilutions to a non-toxic concentration. Cytotoxicity below 0.01 mM was found for doxorubicin, fluoxetine, OH-tamoxifen and tamoxifen. Stock solutions for these substances were therefore diluted in order to determine their estrogenic potential in intact yeast cells.

Out of the 37 pharmaceuticals analysed, 31 showed no induction of β-galactosidase expression in the YES assay. Six pharmaceuticals including cimetidine, fenofibrate, furosemide, paracetamol, phenazone, and tamoxifen showed an induction of β-galactosidase expression. The dose-response curves differed between pharmaceuticals. Near- maximal activity was observed with furosemide (Fig. 1). The metabolite OH-tamoxifen showed a different pattern to the other compounds with only very low induction (Fig. 1). The anti-estrogenic tamoxifen and its metabolite OH-tamoxifen are known to show an induction in the YES, because of their antagonistic and partial agonistic interaction with the hERα. Both compounds were therefore excluded from mixture experiments. Best-fit values for the sigmoidal dose-response fit of the respective substances are given in Table 3. The individual substances do not all reach their maximal induction in the tested concentration range according to the TOP values given in Table 3. The TOP values for the substances showing an estrogenic activity are between 14% (fenofibrate) and 89% (furosemide) of the standard E2.

Furosemide that showed the highest estrogenic activity was subject to a further analysis to test for binding to the hERα. A concentration of 3.13 mM of furosemide, that induced β-galactosidase activity to a level of 65% of the maximal E2-induction, was added to the assay medium. The anti-estrogen OH-tamoxifen was then serially diluted

Table 3: Best-fit values for substances showing an induction of β-galactosidase expression in the YES-assay.

Substance	n	EC_{50} (mM)	log EC_{50}	S.E.M.	Bottom	S.E.M.	Top	S.E.M.	Hillslope	S.E.M.
Cimetidine	16	6.515	0.8139	0.01530	0.01144	0.00148	0.4372	0.01439	2.732	0.1766
Fenofibrate	12	0.6562	−0.1830	0.02150	0.00296	0.00257	0.2496	0.00626	2.121	0.1866
Furosemide	16	1.419	0.1521	0.00581	0.00431	0.00339	1.570	0.01234	1.976	0.0413
Paracetamol	12	25.53	1.407	1.996	−0.00438	0.00062	4.062	58.16	3.612	1.467
Phenazone	12	4.381	0.6416	0.01420	-0.01086	0.001838	0.9506	0.01733	1.382	0.03391
Tamoxifen	16	0.000181	−3.743	0.03754	-0.03412	0.03874	1.239	0.04446	1.201	0.1306
OH-tamoxifen	8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
E2 (standard)	54	1.29×10^{-7}	−6.910	0.00176	0.00760	0.00172	1.772	0.00253	1.741	0.0109

n, number of replicates, S.E.M., standard error of means, n.a. = not available.

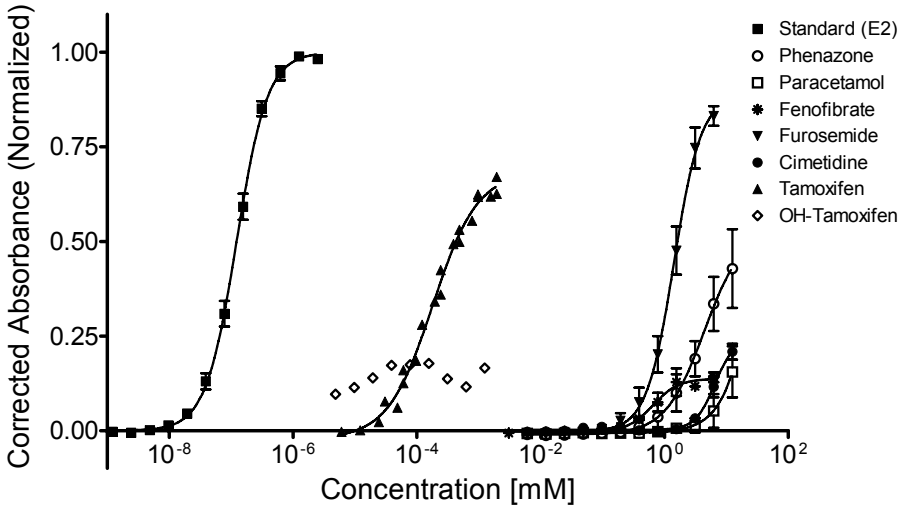

Figure 1: Dose-response curves for the pharmaceuticals showing an induction of β-galactosidase expression in the YES-assay. The E2 standard curve represents the mean of all standard curves obtained with all plates (n = 54). Mean values with SEM for single pharmaceuticals from at least three independent experiments (n=12), anti-estrogens tamoxifen (n = 16) and OH-tamoxifen (n = 8). A sigmoidal dose-response curve could not be fitted for OH-tamoxifen using the four-parameter logistic equation in GraphPad Prism®.

in the concentration range from 10 nM to 10 µM and transferred to the microtiter plates in 5 µL aliquots to block the estrogenic activity of furosemide. A blank row of assay medium containing furosemide, but no OH-tamoxifen describes the response obtained by furosemide alone. Figure 2 shows that the induction caused by furosemide decreases with increasing OH-tamoxifen concentration. Therefore, OH-tamoxifen competing for hERα-binding blocks the estrogenic activity of furosemide in concentrations from about 100 nM in a dose-dependent manner. This is evidence that furosemide actually binds to the hERα in the recombinant yeast for exhibiting β-galactosidase induction.

Pharmaceuticals showing an estrogenic activity were further analysed for anti-estrogenic activity. The assay was carried out as for estrogenicity except for the assay medium containing a single concentration of 8.43×10^{-8} M of E2, which corresponds to the EC65 value producing a submaximal response. An antagonistic interaction with the hERα is indicated by a decreasing β-galactosidase production and demonstrates an anti-estrogenic effect. Microtiter plates in the anti-estrogen assay contained a row of blanks (2.5% DMSO control, the only wells without E2) used for the corrections of the readings, three rows of the anti-estrogen OH-tamoxifen used as standard, and the pharmaceutical to be analysed in quadruplicate. Anti-estrogenicity assays were repeated at least three times. Apart from the expected anti-estrogenic tamoxifen and

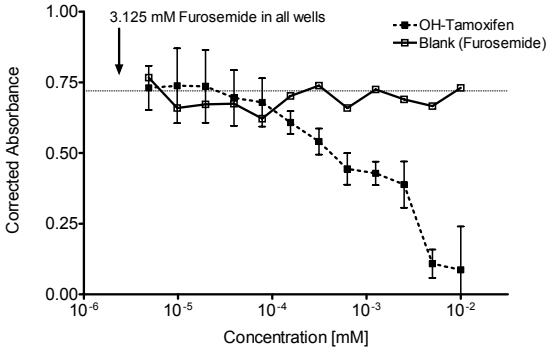

Figure 2: Decreasing estrogenic activity of furosemide with increasing OH-tamoxifen (dotted line) concentrations (n = 9). Furosemide concentration in all wells 3.125 mM. Dotted horizontal line represents the expected absorption reached by furosemide if applied alone at the indicated concentrations.

OH-tamoxifen, none of the five estrogenic pharmaceuticals exhibited an anti-estrogenic activity (data not shown).

Estrogenic activity of compound mixtures

As the steepness of the dose-response curves, best-fit parameters and maximal activities are different between the individual substances, estrogenic activities of pharmaceutical mixtures are difficult to predict. Moreover, the estrogenic activity of the pharmaceuticals did not reach the maximal response of E2. An EC50 for the individual substances cannot be calculated, if the maximal response lines for two substances are not equal, as in the case of cimetidine, fenofibrate, paracetamol and phenazone. Therefore, the EC50 values do not provide a comparable measurement category, as the top values are not the same for all substances. Instead, a concentration value C was defined for every β-galactosidase inducing substance to calculate the required concentration for the various equipotent mixtures.

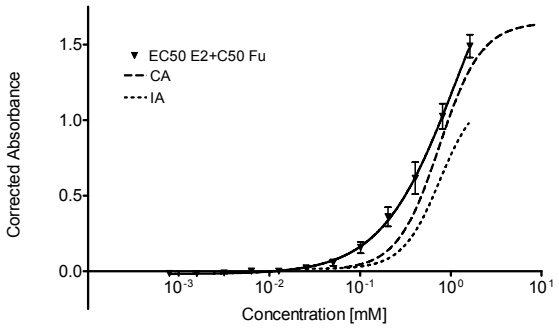

Figure 3: EC50 E2+C50 furosemide mixture with predictions according to the concen-tration addition (CA) and the independent action (IA) concept. Error bars for the measured data are SEM (n = 12).

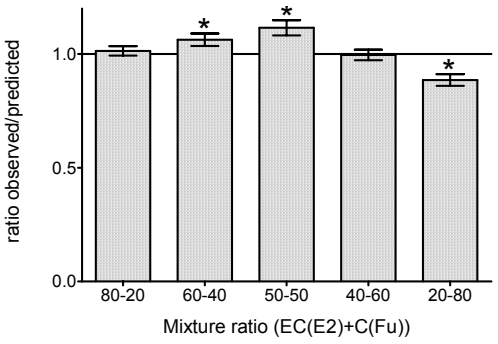

Figure 4: Ratios between measured and expected (CA) absorption values for the highest analysed concentrations of various E2+furosemide mixtures. Stars indicate significant differences between measured and observed values ± SEM. Averages of n = 11-12.

Furosemide is the only pharmaceutical exhibiting a parallel dose-response curve to E2, but the estrogenic activity did not reach maximal values. Combinations of E2 and furosemide were analysed in binary mixtures in various ratios. Figure 3 shows the activities for the equipotent mixture of E2 and furosemide at the concentration at which 50% of the maximal effect for E2 is reached. The fitted curve for various concentrations of the mixture follows more closely the predicted CA curve than the predicted IA curve, which were calculated according to the formulas (3) and (4) (see Materials and Methods). As shown in figure 4, the measured mean for the highest concentration follows the prediction at the EC80+C20 and EC40+C60 mixture, but differs in the other mixture ratios. The statistics in Table 4 demonstrates that the CA model adequately predicts the values for the E2 and furosemide EC80+C20 and EC40+C60 mixture, but not for EC20+C80.

Figure 5 shows the analysis of the mixture effects using the isobole method (Kortenkamp and Altenburger 1998). The line connecting the iso-effective dose for E2 on the x- axis and furosemide on the y-axis predicts that the combination is additive according to the CA model. Concave down-isoboles for mixtures indicate that mixture yield the same effect at lower concentrations and the compounds therefore act more

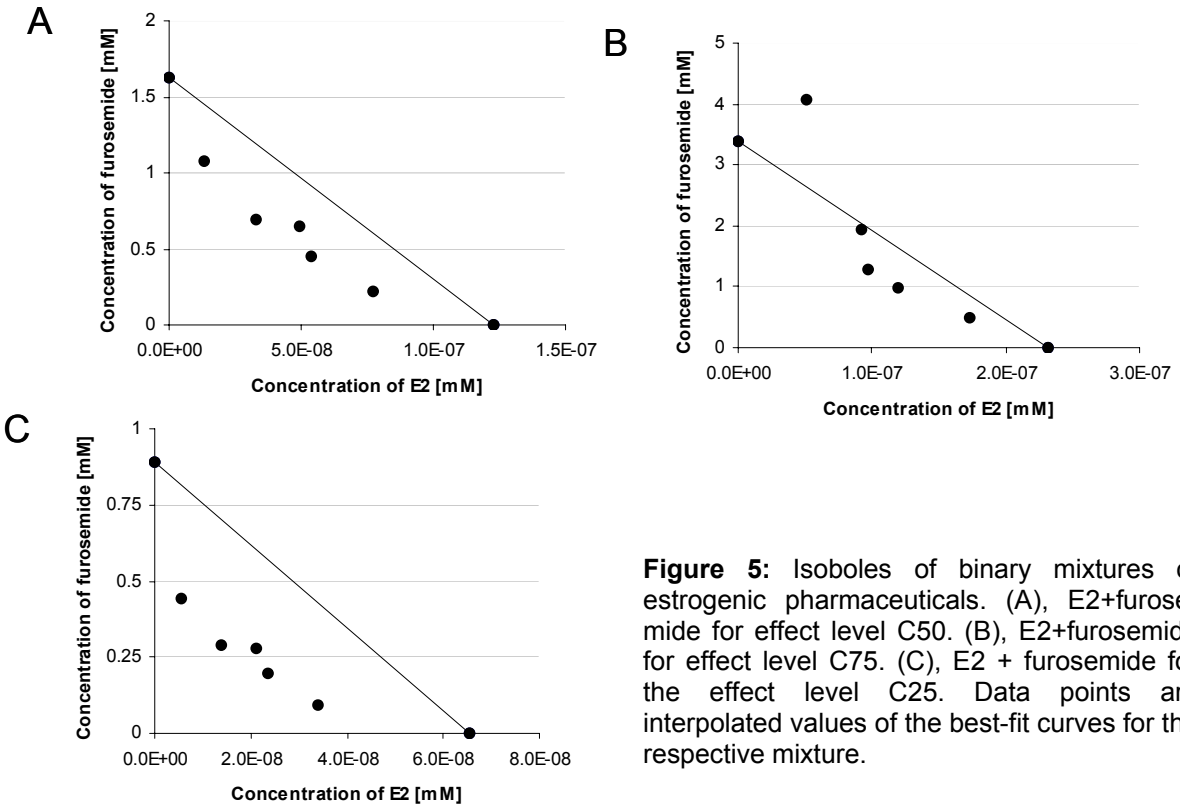

Figure 5: Isoboles of binary mixtures of estrogenic pharmaceuticals. (A), E2+furosemide for effect level C50. (B), E2+furosemide for effect level C75. (C), E2 + furosemide for the effect level C25. Data points are interpolated values of the best-fit curves for the respective mixture.

than additive or synergistically. Concave-up isoboles indicate antagonistic mixtures. At the effect level C25 and C50 the binary mixture of E2 and furosemide seem to act synergistically. At the effect level C75, the data points scatter along the additivity line. Therefore, the combined mixture effects of E2 and furosemide follow the CA model with a trend to synergism, depending on the effect level.

The equipotent binary mixtures of furosemide and phenazone at the C20 and C25 levels follow the CA model predictions more closely than the IA prediction, but the difference between the predicted values is very minor (Fig. 6A, 6B). At lower concentrations activities are higher than predicted values according to both the CA and IA model, which is similar to the binary mixture of furosemide and E2. At the highest measured concentrations, measured and predicted values do not differ (Fig. 6C).

Various combinations of two to five individual pharmaceuticals were mixed at their C01 and C10 levels. The results shown in figure 7 indicate that even for binary mixtures it is not always possible to predict the outcome of the experiments using either the CA model or the IA model. The estimates based on the CA model and IA model are very similar for the mixtures analysed, and a distinction between the different models is not always possible. Seven mixtures followed the prediction of the CA model, but 8 mixtures exhibited dramatically higher activities than expected. Of the six mixture combinations at the C01 level, three followed the CA model prediction, whereas one

Table 4: One-sample t-test for the highest concentrations of all mixtures.

Mixture	Mean	n	Estimate CA	Estimate IA	S.D.	t-Value CA	t-Value IA	P-value (n − 1)	Decision (P = 95%) CA	Decision (P = 95%) IA
E2 + Fu $C_{50} + C_{50}$	1.490	12	1.336	0.988	0.1309	4.08	13.28	2.201	Significant	Significant
E2 + Fu $C_{80} + C_{20}$	1.512	11	1.484	1.27	0.0800	1.16	10.04	2.228	Not significant	Significant
E2 + Fu $C_{60} + C_{40}$	1.454	11	1.349	1.020	0.1056	3.29	13.61	2.228	Significant	Significant
E2 + Fu $C_{40} + C_{60}$	1.340	12	1.349	1.020	0.1100	0.28	10.08	2.201	Not significant	Significant
E2 + Fu $C_{20} + C_{80}$	1.318	12	1.467	1.270	0.1056	4.88	1.58	2.201	Significant	Not significant
C_{20} FuPh	0.741	20	0.667	0.614	0.2535	1.31	2.25	2.093	Not significant	Not significant
C_{25} FuPh	0.773	19	0.773	0.704	0.2516	0.01	1.19	2.101	Not significant	Not significant
C_{01} FuPhCiFf	0.226	16	0.196	0.080	0.0827	1.44	7.07	2.131	Not significant	Significant
C_{01} FuPhCiFfPa	0.617	12	0.315	0.120	0.1296	8.06	13.28	2.201	Significant	Significant
C_{10} FuPhCiFfPa	0.877	20	0.320	0.481	0.2997	8.30	5.90	2.093	Significant	Significant
C_{10} FuCi	0.406	10	0.403	0.335	0.1257	0.07	1.78	2.262	Not significant	Not significant
C_{10} FuFf	0.327	13	0.255	0.334	0.1436	1.81	0.19	2.179	Not significant	Not significant
C_{10} FuPa	0.559	10	0.469	0.205	0.1279	2.21	8.74	2.262	Not significant	Not significant
C_{10} CiFf	0.287	6	0.251	0.335	0.4841	0.18	0.24	2.571	Not significant	Not significant
C_{10} FuCiFf	0.768	12	0.288	0.457	0.1761	9.45	6.12	2.201	Significant	Significant
C_{10} FuPhFf	0.641	7	0.161	0.457	0.0944	13.43	5.15	2.447	Significant	Significant
C_{10} PhCiFf	0.637	11	0.287	0.457	0.1708	6.79	3.49	2.228	Significant	Significant
C_{10} PhCiFu	0.855	10	0.496	0.457	0.1230	9.22	10.23	2.262	Significant	Significant
C_{01} FuFf	0.078	12	0.116	0.050	0.0339	3.86	2.87	2.201	Significant	Significant
C_{01} FuCi	0.087	14	0.086	0.050	0.0733	0.05	1.87	2.160	Not significant	Not significant
C_{01} FuPa	0.157	10	0.145	0.050	0.0468	0.82	7.21	2.262	Not significant	Significant
C_{01} CiFf	0.146	10	0.082	0.050	0.0761	2.65	3.99	2.262	Significant	Significant
NOEC	0.067	8	0.0252	1.43E-02	0.0177	6.71	8.44	2.365	Significant	Significant

Given are mean values of n experiments, estimates including standard deviations (S.D.), and P-values. The decision whether the mean differs significantly from the prediction is based on the two-sided t-table for P = 95%. Fu: furosemide; Ph: phenazone; Ci: cimetidine; Ff: fenofibrate; Pa: paracetamol; NOEC: no observed effect concentration ($C_{0.05}$).

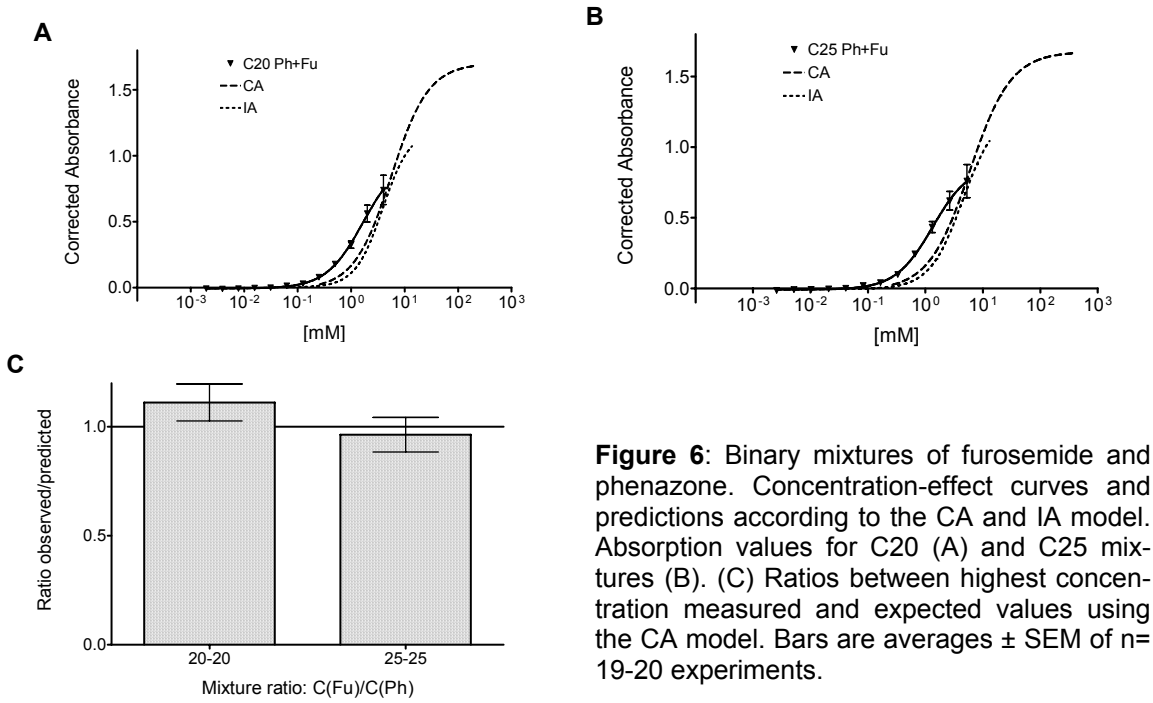

Figure 6: Binary mixtures of furosemide and phenazone. Concentration-effect curves and predictions according to the CA and IA model. Absorption values for C20 (A) and C25 mixtures (B). (C) Ratios between highest concentration measured and expected values using the CA model. Bars are averages ± SEM of n= 19-20 experiments.

mixture (furosemide and fenofibrate) showed lower activity than expected. The mixture of fenofibrate and cimetidine, and the mixture comprising of five pharmaceuticals at their C01 level had much higher activities than expected.

The C10 mixtures of two active pharmaceuticals did not differ from the CA model. In contrast, the effects of the C10 mixture comprising of three and four pharmaceuticals (furosemide, phenazone, cimetidine and fenofibrate) were dramatically higher than expected. The activity of mixtures containing three compounds is underestimated by both the CA and the IA model.

The mixture of four pharmaceuticals, cimetidine, fenofibrate, furosemide and phenazone, showed a dramatically higher activity than expected from the model prediction, when mixed at their C0.05 level (NOEC level) (Fig. 7). The pharmaceuticals mixed at extremely low –effect concentrations lead to a significant response.

To compare the mean of measured values at the highest concentration to the hypothetical mean calculated using the CA or the IA model, respectively, a statistical evaluation using the one-sample t-test was performed (Table 4). P values were taken from the two-sided t-table for P=95%. There was no significant difference from the CA prediction in eleven out of 23 analysed mixtures. In twelve mixtures, the observed values deviate significantly from the CA model prediction (Table 4).

The results of the various mixture experiments demonstrate that the limitations given by the diverse shapes of the compound's concentration-response curves and maximal

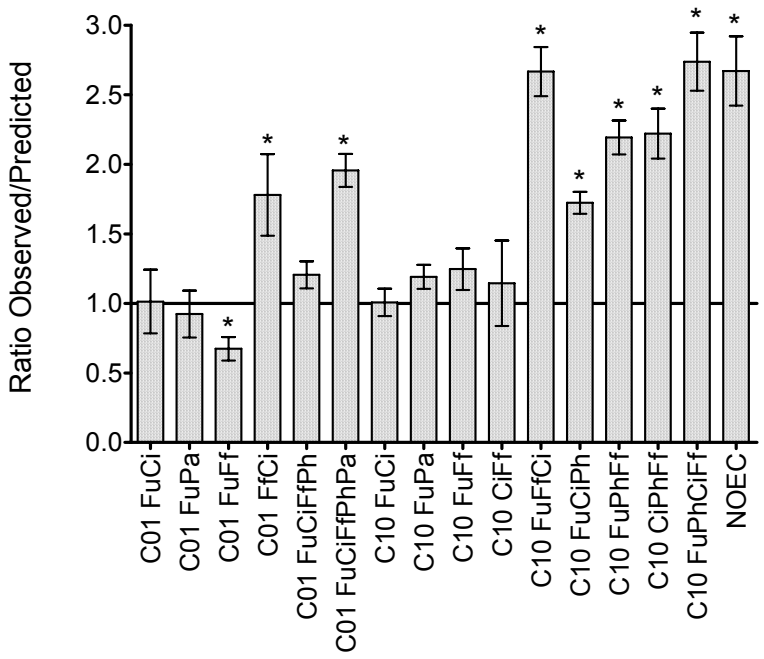

Figure 7: Ratios between measured and expected (CA model) absorption values for various mixtures at the highest concentrations. Stars indicate significant differences between observed and predicted values. Measured values are highest analysed concentrations. Fu, furosemide; Ci, cimetidine; Pa, paracetamol; Ff, fenofibrate; Ph, phenazone; NOEC, no observed effect concentration. Error bars indicate SEM.

activities lead to results that deviate from the CA model predictions. For very low effect levels (C01, C0.05) we observed dramatically higher activities than expected. This clearly indicates that accurate predictions of mixture effects are only possible, if the shape of the concentration-response curves of single compounds are almost equal, and when the maximal activity is almost reached.

Discussion

In this work, we demonstrate the *in vitro* estrogenic activity of five pharmaceuticals that occur in the aquatic environment. We applied the YES assay which is a robust and simple assay providing important information on the binding of estrogenic chemicals to the hERα and ERE activation (Kunz *et al.* 2004; Routledge and Sumpter 1996). Out of the 37 pharmaceuticals, furosemide, phenazone, fenofibrate, cimetidine and paracetamol showed weak estrogenic activity (Fig. 1). Tamoxifen and furosemide exhibited a dose-response curve with almost full efficacy, whereas the other compounds did not reach maximal activity. Furosemide was demonstrated to exhibit estrogenic activity via binding to the hERα (Fig. 2). This pharmaceutical was about 10^7

times less potent than E2. Compared to 4-nonylphenol and bisphenol A, two substances with a known estrogenicity (Sohoni and Sumpter 1998), furosemide was about 1,000 times less potent. Furosemide, but also the other pharmaceuticals including phenazone, cimetidine, fenofibrate and paracetamol can be classified as very weak hERα agonists, as they exhibited no anti-estrogenic activity.

The estrogenic potential of a substance is hardly predicted by its structure alone and the precise requirements for estrogenicity are not yet fully understood (Kunz *et al.* 2004; Routledge and Sumpter 1996; Sumpter and Johnson 2005). The pharmaceuticals having estrogenic activity have no structural resemblance to natural estrogens (Fig. 8). The only molecular feature resembling those of other known estrogens is found with paracetamol that bears a phenol moiety.

Many of the estrogenic compounds exert their effects via interaction with the estrogen receptor, as the estrogenic pharmaceuticals identified in this study. Whether or not the *in vitro* activity is paralleled by activity *in vivo* remains to be shown. In some cases, *in*

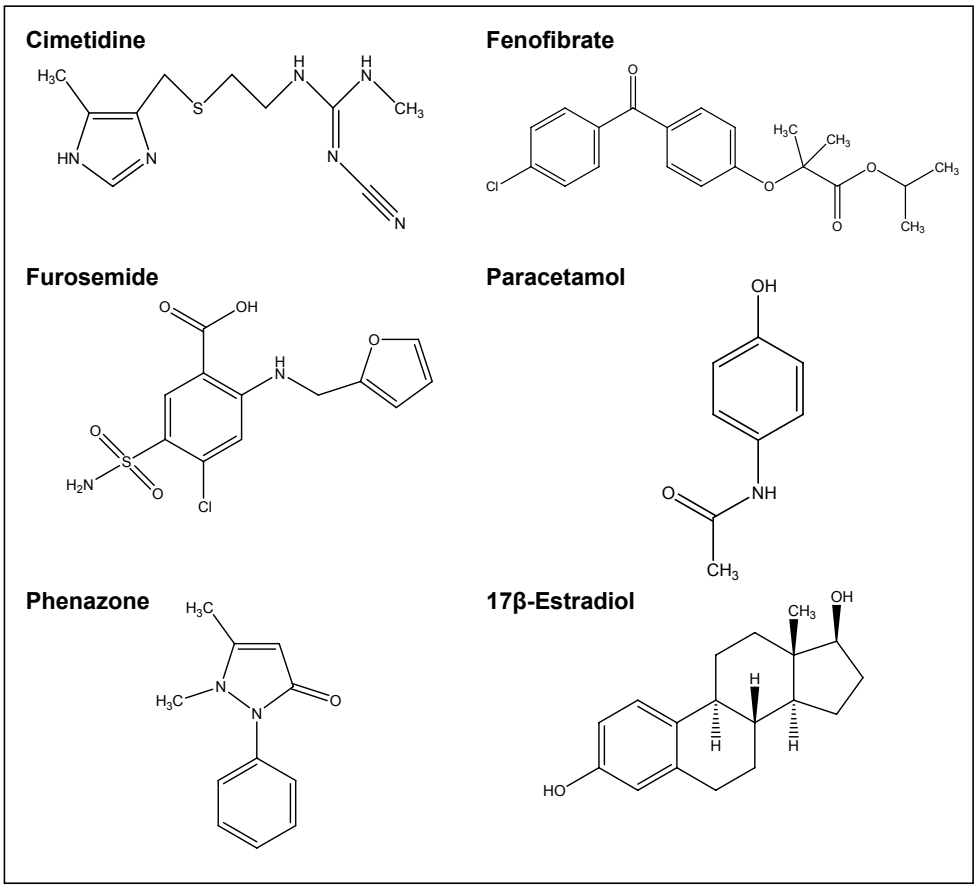

Figure 8. Chemical structures of estrogenic pharmaceuticals identified in the YES-screen and of 17β-estradiol.

vitro and *in vivo* activities clearly correlate, in other cases, *in vitro* activity cannot be extrapolated to the *in vivo* activity (Kunz *et al.* 2004). Only *in vivo* studies will show whether the estrogenicity is paralleled by *in vivo* activity.

The half-maximal estrogenic activity found in the YES-assay was in the range of 0.66-25.53 mM, or hundreds of μg/L to g/L. Typical environmental concentrations in STP effluents are in the ng/L to μg/L range (Fent *et al.* 2006; Ternes *et al.* 2004). Furosemide was found in all monitored wastewaters in Italy (Castiglioni *et al.* 2004). The daily loads in the STP influent was 277 mg/1,000 inhabitants and in the effluent 195 mg/1,000 inhabitants with an average removal in the STP of 15%. In surface waters, the daily load in Italy was 66 mg/1,000 inhabitants, which indicates a widespread occurrence of furosemide.

Table 5 compares the half-maximal values (EC50) found in the YES-assay of the present study with the maximal environmental concentrations reported. Except for E2, which was used as a standard throughout the study, and for tamoxifen, the EC50 values are a factor of 10^5 to 10^6 higher than the maximal environmental concentrations. This indicates that the *in vitro* activities of these pharmaceuticals are weak. An estrogenic effect of the single pharmaceuticals that show an estrogenic potential in the YES-assay may therefore not be expected at environmental concentrations. Still, for hazard and risk assessments, it is important to know the estrogenic potential of pharmaceuticals that are ubiquitously found in the environment and may contribute to the complex mixtures.

Comparisons of effect concentrations in the YES-assay with environmental concentrations can be regarded as a first hint at possible environmental hazards and risks. However, only *in vivo* experiments can account for metabolism and additional effects in animals and provide direct information on the estrogenic potential of the pharmaceuticals in an organism. It should also be noted that the ratios between effect concentrations found in the conducted *in vitro* studies and the current environmental concentrations are very high.

Table 5: Ratios between observed EC50 values in the YES-assay and maximal environmental concentrations found in the literature for STP effluents.

Pharmaceutical	EC_{50} (mM)	Concentration (g/L)	Maximal environmental concentration (g/L)	Factor (Con/max Envir. Conc)	Source
Cimetidine	6.52	1.64	5.80E−07[a]	2.83E+06	[1]
Fenofibrate	0.66	0.24	1.20E−06	1.97E+05	[6]
Furosemide	1.42	0.47	–		[35]
Paracetamol	25.53	3.86	1.00E−05	3.86E+05	[1]
Phenazone	7.74	1.46	3.08E−06	4.73E+05	[37]
Tamoxifen	1.90E−04	7.06E−05	4.20E−08	1.68E+03	[12]

[a] Concentration in wastewater.

Effects of mixtures

Aquatic organisms are generally exposed to a complex mixture of xenobiotics, particularly in environments contaminated by wastewater. Generally, the individual assessment of the hazard posed by single chemicals indicates a negligible risk, with the exception of E2 and EE2 (Sumpter and Johnson 2005). However, this does not account for the potential of endocrine active substances to act in combination. This may lead to the underestimation of hazards that exist in the actual exposure situation, resulting in erroneous assumption of absence of risk. The assessment of the toxicity or estrogenic activity of mixtures cannot be calculated by simply adding the effects of the mixture components when applied singly. As the YES-assay is based on the binding of substances to hERα, the concept of CA is likely to be applicable. Concentration addition is widely regarded as the appropriate concept for mixtures, whose components act in a similar mode of action such as on a defined receptor (Backhaus *et al.* 2004). The IA model in contrast is usually applied when mixtures consist of dissimilarly acting substances (Kortenkamp and Altenburger 1998). Both concepts require that all mixture components are known as well as their respective concentrations and effects, which holds true in a laboratory assay, but is difficult or even unrealistic to apply in an environmental sample where unknown chemicals in unknown quantities are present.

The activity of binary mixtures of furosemide and E2 (Fig. 4, 5), and of furosemide and phenazone (Fig. 6) are estimated accurately by the CA model. The mixture combinations of E2 and furosemide (EC80 + C20 and EC40 + C60) are better estimated by the CA than IA model, as expected for similarly acting substances. However, for the analysed mixtures, the estimates discriminate only little between the CA and IA model. The isoboles in figure 5 indicate that the two compounds interacted in an additive manner, although at two mixture ratios (C25, C50) synergistic interaction is suggested. Mixtures of furosemide and phenazone also followed the CA model.

Various combinations of two to five individual pharmaceuticals were mixed at their C01 and C10 levels (Fig. 7). The estimates based on the CA model and IA model are very similar for the mixtures analysed, and a distinction between the different models is not always possible. The mixture of four pharmaceuticals at their NOEC level showed a dramatically higher activity than predicted by the models. Seven mixtures followed the prediction of the CA model, but 8 mixtures exhibited dramatically higher activities than expected. The results indicate that even for binary mixtures it is not always possible to

predict the outcome of the experiments using the CA model due to the following reasons.

The concentrations of the pharmaceuticals in the analysed mixtures are very high as compared to E2. Moreover, pharmaceuticals did not reach maximal responses and dose-response curve were not identical, as required for the CA model. The curves of the single compounds used for the interpolations of the respective concentrations might be too unreliable at low effect concentrations, what makes predictions difficult. Therefore, the calculated values may deviate dramatically from the real mixture effects. These are the reasons why mixture effects of binary mixtures of pharmaceuticals at the C01 and C10 level and of mixtures of up to five weakly estrogenic pharmaceuticals were hardly predictable. Similarly, (Rajapakse *et al.* 2004) have also found deviations from expected mixture effects estimated by CA in mixtures of five and six estrogenic chemicals including genistein, 4-nonylphenol, 4-tert-octylphenol, 17β-estradiol, 17α-ethinylestradiol, and bisphenol A. These were interpreted as interactions between the chemicals, which cannot be applied to our mixture experiments.

Therefore, the results of the various mixtures analysed at different effect levels indicate that it is difficult or even not possible to accurately estimate the combined effect of low concentrations of weakly estrogenic compounds having only minimal efficacy. This emphasises that accurate predictions of mixture effects can only be made if the individual compounds exhibit identical shapes in their concentration-response curves and if they have maximal activity. When these conditions are not fulfilled, estimates are inaccurate or at least very difficult. However, compounds in the environment have such diverse concentration-effect relationships and therefore, our mixture experiments may mimic realistic environmental situations. The lack of appropriate predictions may also suggest limitations of this mixture concept.

Despite these difficulties our data demonstrate that pharmaceuticals, which are mixed at extremely low concentrations such as at the NOEC or at the C01 level, exhibit a marked activity. The results clearly indicate that the estrogenic compounds mixed at extremely low-effect concentrations lead to a significant response. The concept that a mixture of estrogenic chemicals, each of which is present at very low-effect concentration, can induce a significant response (Cromlish and Kennedy 1996; Stumpf *et al.* 1999) is confirmed by our experiments with pharmaceuticals. It gives further evidence to the observation that very low concentrations of estrogenic chemicals,

which appear to have little or no effect on their own, are nonetheless biologically active, and when combined with other estrogenic compounds, may exert effects.

The interactions of estrogenic pharmaceuticals with other estrogens present in wastewater indicate that the principle of CA holds true for multicomponent mixtures of estrogenic compounds *in vitro*. Whether this translates directly to *in vivo* activity as with other estrogenic compounds remains to be shown in forthcoming experiments. The additive nature of the combined effects demonstrated that both components contribute to the overall effect of a mixture. This implies that the overall effects will exceed the highest individual effect of the mixture components. This is of particular importance for the environmental hazard and risk assessment of pharmaceuticals, because it indicates that concentrations of single chemicals that show no effect when applied singly may provoke substantial effects when acting in combination (Cromlish and Kennedy 1996).

The European Medicines Evaluation Agency (EMEA) published a draft guideline on the environmental risk assessment of medicinal products for human use (EMEA 2005). This guideline applies mainly to new active substances and requires ecotoxicological investigations. In the European Union, a new system is planned for the assessment of environmental risks and effects on human health for a wide range of chemicals that include pharmaceuticals as well. The registration, evaluation and authorisation of chemicals (REACH) regulation would take into account not only new substances, but also established chemicals that are produced in large amounts. The *in vitro* analysis of estrogenic activity as shown in this study is a possible strategy for assessing pharmaceuticals within this framework.

Experiments with mixtures of estrogenic substances showed that the prediction of the activity of complex mixtures consisting of substances that exert only a weak effect when applied singly are very difficult. Even at low concentrations, the effects may be much higher than expected from the concentration-response curves of the single compounds. In aquatic environments, many diverse estrogenic substances such as UV-filters, alkylphenols, bisphenol A, phthalates or steroid hormones, to mention only a few, are ubiquitous, particularly in sewage contaminated systems. Many of them show incomplete concentration-response curves. It would be interesting to further test the CA concept for reliability of the results presented in this study. Moreover, analysis of mixtures of a larger set of different pharmaceuticals would allow drawing a more realistic picture of the exposure situation in the environment.

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Abbreviations

ABCX	ATP-Binding Cassette belonging to the family X
ATP	Adenosine Triphosphate
BCRP	Breast Cancer Resistance Protein
CYP	Cytochrome P450
FAO	Fatty Acyl coenzyme A Oxidase
EC50	half-maximal Effect Concentration
LogD	Partition Coefficient correlated to a specific pH-value
MDR	Multidrug Resistance
MDR1	P-glycoprotein1 (ABCB1)
MRP1	Multidrug Resistance-related Protein 1 (ABCC1)
MRP3	Multidrug Resistance-related Protein 3 (ABCC3)
MTT	Mitochondrial Tthiazolyl blue Tetrazolium bromide
MXR	Multixenobiotic Resistance
NR	Neutral Red
P-gp1	P-glycoprotein 1 (ABCB1, MDR1)
PLHC-1	*Poeciliopsis lucida* Hepatocellular Carcinoma cell line
PLHC-1/dox	Doxorubicin-resistant subclone of PLHC-1
PLHC-1/wt	Wild type PLHC-1
PPAR	Peroxisome Proliferator-Activated Receptors
PPRE	Peroxisome Proliferator Response Elements
RTG-2	Rainbow Trout Gonadal cell line
RxR	Retinoid X Receptor

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Curriculum Vitae

Name	Caminada
First Name	Daniel
Date of Birth	23rd of January 1978
Place of Birth	Chur/GR
Hometown	Vrin/GR
Nationality	Swiss

EDUCATION

2004-2007	**University of Zurich -** Institute of Plant Biology PhD Project: Investigation and assessment of effects of pharmaceuticals on aquatic organisms in an *in vitro* model system.
Okt 1999 – Mar 2004	**ETH Zurich** Master's degree in biochemistry and molecular biology
Okt 1998 – Jul 1999	**ETH Zurich** Studies in physik
Aug 1991 – Jun 1998	**High school Graubünden** Matura Typus B

SCIENTIFIC COMMUNICATIONS

2007	Platform presentation at the international Meeting "ABC transport proteins in environmental health and toxicology", Siena (I)
2007	Platform presentation and poster at PRIMO14, Florianopolis (BR)
2006	Poster at SETAC Europe 2006, 16. European conference, Den Haag (NL).

PEER-REVIEWED PUBLICATIONS

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